The University of Alberta

REGULATION OF CARDIAC ENERGY METABOLISM AND ISCHEMIA REPERFUSION INJURY BY STRESS RESPONSIVE PROTEIN KINASES

by

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DEDICATION

This thesis is dedicated to my parents, Harbhajan and Balwant Jaswal.

ABSTRACT

Optimizing cardiac energy substrate metabolism in the ischemic and reperfused myocardium represents a novel mechanism to limit and/or ameliorate ischemia-reperfusion (IR) injury, and thus enhance the recovery of post-ischemic function. Attenuating the rate of glycolysis improves the coupling between glycolysis and glucose oxidation, and so reduces the rate of proton (H^+) production, a facet of myocardial carbohydrate metabolism that is central to the cardioprotective effects of adenosine. Conversely, in select experimental models, the cardioprotective effects of adenosine are lost, and are associated with an uncoupling of glycolysis and glucose oxidation, and an acceleration of the rate of H^+ production. The cellular signaling events responsible for IR injury and these metabolic effects of adenosine remain to be characterized. The stress-responsive protein kinases, AMPK and p38 MAPK are implicated in IR injury as well as the regulation of glucose uptake, and may influence myocardial glucose metabolism, and thus represent novel pharmacological targets to attenuate IR injury.

This study characterized the role of p38 MAPK in IR injury following hypothermic ischemia, as well as the roles of p38 MAPK and AMPK in the adenosine-induced acceleration of glycolysis and H^+ production in hearts stressed by transient ischemia (2 X 10-min I / 5-min R). The inhibition of p38 MAPK following hypothermic ischemia was cardioprotective. During aerobic perfusion, the adenosine-induced activation of AMPK was associated with an acceleration of the rate of glycolysis. This was independent of increased glucose uptake, but was accompanied by a suppression of the rate of glycogen synthesis. Inhibition of p38 MAPK prevented the activation of AMPK, and subsequent uncoupling of glycolysis and glucose oxidation by relieving the

suppression of glycogen synthesis. In stressed hearts subjected to severe IR, inhibition of p38 MAPK decreased the activity of AMPK, and improved the coupling between glycolysis and glucose oxidation by promoting glycogen synthesis, effects accompanied by enhanced post-ischemic function.

Thus, data described in these studies indicates that p38 MAPK and AMPK are involved in the regulation of glucose utilization at the level of the balance between glycolysis and glycogen synthesis, and that these kinases may represent novel targets to limit myocardial IR injury.

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Chapter 1

INTRODUCTION

Myocardial ischemia occurs when coronary blood flow is inadequate, and hence the oxygen supply to the myocardium is not sufficient to meet oxygen demand. The manifestations of myocardial ischemia are dependent upon the nature, and severity of the ischemic episode and the subsequent re-establishment of flow (reperfusion); consequences include changes in cardiac ultrastructure, functional deficits, and metabolic alterations.

In Canadian society there has been marked decline in the number of deaths due to acute myocardial infarction (AMI) (21,962 deaths in 1997 compared to 27,656 deaths in 1984) attributed to improved therapies [1]. As a result of improved AMI survival rates the number of individuals living with cardiovascular diseases (e.g. angina pectoris) has increased [1]. In addition to accounting for 74,626 deaths in 2002, cardiovascular disease also carries an enormous economic burden, costing the Canadian economy over 18 billion dollars per year. Ischemic heart disease(s) comprises a major portion of both the mortality and economic burden of overall cardiovascular disease.

Classically the treatment of ischemic heart disease has focused on restoring the balance between oxygen (O_2) supply and demand (increasing O_2 supply and/or decreasing O_2 demand) such that O_2 supply is sufficient to meet oxygen demand. An emerging and novel intervention to treat ischemic heart disease is the manipulation of myocardial energy substrate metabolism and its biochemical mediators such that the efficiency of converting the hydrolysis of adenosine tri-phosphate (ATP) into contractile work is maximized, and oxygen use is reduced.

Adenosine is a pharmacological agent that limits myocardial ischemic injury in part by altering cardiac carbohydrate metabolism. However, the biochemical mediators

underlying the adenosine-induced alterations in carbohydrate metabolism and adenosine-induced cardioprotection have not yet been clearly defined. Thus as described in this thesis a further understanding of the changes in myocardial energy substrate metabolism and the biochemical mediators responsible for such changes in the setting of myocardial ischemia-reperfusion (IR) is essential for elucidating novel and viable pharmacological targets to limit IR injury.

Myocardial Ischemia-Reperfusion Injury

1.1 Development of ischemia-reperfusion injury

1.1.1 Pathophysiology of ischemia-reperfusion injury

Myocardial IR injury results from the impairment of coronary blood supply attributed to occlusion due to thrombosis or other alterations of coronary atherosclerotic plaque architecture [2]. As the heart extracts 70-80% of the molecular O_2 per unit of blood delivered [3] myocardial ischemia refers to a situation where oxygen availability is no longer sufficient to meet the oxygen requirements of the heart [4]. In the ischemic myocardium there is a rapid loss of contractile force, a depletion of high energy phosphates, and disturbances of ionic homeostasis [5]. As ischemia reduces the production of ATP from mitochondrial oxidative phosphorylation, the heart must produce ATP anaerobically via glycolysis during periods of prolonged ischemia. The inreased reliance on glycolysis for ATP during ischemia leads to the accumulation of lactate and protons (H^+) (glycolytic intermediates) causing intracellular acidosis, which contributes to ischemic injury.

Coronary reperfusion via thrombolysis, percutaneous coronary intervention, or coronary artery by pass grafting is established for the management of AMI; however, the restoration of flow to the previously ischemic myocardium produces a spectrum of pathophysiological effects collectively referred to as reperfusion injury [6]. Post-ischemic dysfunction can be manifest as both reversible and irreversible injury, ranging from cardiac dysrhythmias and cardiac stunning, to myocyte death in the form of necrosis and apoptosis (programmed cell death). The molecular and cellular mechanisms implicated in myocardial IR injury include oxidative stress and the dysregulation of ionic homeostasis.

1.1.2 Pathogenesis of Ischemia-Reperfusion Injury – Reactive Oxygen Species

Reperfusion of the previously ischemic heart which has accumulated anaerobic metabolites results in the production of reactive oxygen species (ROS) including the superoxide anion ($O_2^{\cdot -}$) and hydroxyl radical ($OH^{\cdot}$), which are recognized as crucial mediators of myocardial IR injury [7]. The major sources of ROS are the mitochondrial electron transport chain and enzymes including xanthine oxidase, NADPH oxidase, cyclooxygenase, and lipoxygenase [8].

The complete reduction of O_2 via the mitochondrial electron transport chain is coupled to the oxidation of metabolic fuels for the generation of ATP via oxidative phosphorylation. Under normal/aerobic conditions a small percentage (1-4%) of available oxygen is not reduced, and forms $O_2^{\cdot -}$ at complex I (NADH coenzyme Q reductase/ubiquinone oxidoreductase) and complex III (ubiquinole cytochrome C reductase) [9]. However, this percentage increases dramatically during ischemia and reperfusion as cellular hypoxia decreases the activity of complex IV (cytochrome

oxidase) thereby increasing the formation and release of ROS from complexes I and III [7]. The mitochondrial electron transport chain is believed to be the primary intracardiac source of ROS [10,11].

Although the contribution of xanthine oxidase to ROS production following IR in the human heart remains controversial, it is a significant contributor to ROS formation in the canine and rat heart [12,13]. In ischemic tissue, the enzyme xanthine dehydrogenase is converted to xanthine oxidase, a potent generator of $O_2^{\cdot-}$ and hydrogen peroxide (H_2O_2) from the oxidation of xanthine and hypoxanthine to form uric acid [14]. The enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is responsible for $O_2^{\cdot-}$ generation via the "respiratory burst" in activated neutrophils and leukocytes which represent an important extracardiac source of ROS [15]. In addition to NADPH oxidase, neutrophils and leukocytes also utilize the metabolism of arachadonic acid via the cyclooxygenase and lipoxygenase pathways to generate ROS.

The oxidative modification of cardiac proteins contributes to impaired contractile function in the post-ischemic period. The oxidation of sulfhydryl groups from both ventricular and mitochondrial homogenates is associated mechanical dysfunction during reperfusion [16]. Specifically, the oxidation of critical sulfhydryl groups of contractile proteins including actin, desmin, and tropomyosin appears to contribute to myocardial IR injury [17].

Peroxidative damage of phospholipid membranes can interfere with selective permeability and thus impair the function of various cellular organelles [18]. Cardiolipin, an inner mitochondrial membrane phospholipid that contributes to optimal mitochondrial function is subject to peroxidation following ischemia and reperfusion, which can

subsequently impair the activities of complex I and complex III [19,20,21]. As result of cardiolipin peroxidation, mitochondrial function is impaired, and can contribute to a further exacerbation of ROS production during reperfusion following ischemia thus initiating a vicious cycle of ROS production and further oxidative damage.

1.1.3 Pathogenesis of Ischemia-Reperfusion Injury – Dysregulation of Ionic Homeostasis and Ca^{2+} Overload

In addition to lipid peroxidation, ROS species can also interfere with the function of membrane ion channels and ion exchange mechanisms via oxidative modification of protein structure and thus compromise ionic homeostasis [22]. Of particular importance to the cardiac myocyte is the ability of ROS to inhibit the Na^+/K^+ ATPase which is responsible for the extrusion of three Na^+ ions in exchange for 2 K^+ ions and is crucial in regulating resting membrane potential [23]. Impaired function of the Na^+/K^+ ATPase results in intracellular Na^+ overload, and the subsequent reverse mode activation of the Na^+/Ca^{2+} exchanger, such that 3 Na^+ ions are exchanged for 1 Ca^{2+} ion, which contributes to Ca^{2+} overload following IR [24]. Furthermore, ROS can also impair the activity of the sarcoplasmic Ca^{2+} ATPase, which is responsible for the reuptake of Ca^{2+} following myocyte contraction, and thus further aggravate Ca^{2+} overload [25].

As Ca^{2+} is required for cardiac muscle contraction, it is a major determinant of the pathophysiology of post-ischemic contractile dysfunction, via mechanisms involving decreased responsiveness of the contractile proteins to activator Ca^{2+} as well as the activation of Ca^{2+} dependent proteases (calpains) [26]. Specifically, contractile proteins can be subjected to the oxidation of thiol moieties in the post-ischemic period, which may result in impaired responsiveness to Ca^{2+}, suggesting that myocardial IR injury induced

by ROS and Ca^{2+} occurs in a manner that is not mutually exclusive [27]. Calpains are activated by myocardial IR [28], and are implicated in the proteolytic degradation of intracellular proteins including desmin, α-actinin, and spectrin [29] as well as the contractile proteins troponin I [30], and troponin T [31]. Ca^{2+} handling proteins of the sarcoplasmic reticulum, including the Ca^{2+}ATPase, ryanodine receptors (Ca^{2+} release channels), and phospholamban are also subject to degradation by calpains, and thus contribute to the dysregualtion of Ca^{2+} homeostasis which in part underlies myocardial IR injury [32].

1.2 Severity of Myocardial Ischemia-Reperfusion Injury

1.2.1 Reversible Ischemia-Reperfusion Injury

Reversible myocardial IR injury refers to the manifestation of post-ischemic mechanical dysfunction in the absence of irreversible tissue injury in the form of myocyte death either via apoptosis or necrosis. Disturbances in ionic homeostasis contribute significantly to the development of IR injury by promoting a pro-arrhythmic phenotype, which itself is a major contributor to cardiovascular morbidity and mortality. Myocardial stunning is also a major component of reversible IR injury. Myocardial stunning refers to persistent contractile dysfunction in the post-ischemic period in the absence of myocyte loss and despite the restoration of normal to near-normal coronary flow [18]. The persistent contractile dysfunction that characterizes myocardial stunning is reversible with a time course ranging from hours to days [26]. As patients with various forms of ischemic heart disease experience spontaneous reperfusion attributed to the lysis of coronary thrombi or the release of coronary spasm, myocardial stunning may contribute to the morbidity associated with ischemic heart disease [33]. As patients with ischemic heart disease may experience repetitive episodes of ischemia and reperfusion, myocardial stunning may

become chronic and induce a state of myocardial hibernation, characterized by a reversible contractile deficit accompanied by a deficit in coronary flow [34].

1.2.2 Irreversible Ischemia-Reperfusion Injury

Prolonged ischemia exerts irreversible injury causing cardiac myocyte death/infarction, the extent of which is proportional to the duration of the interval between coronary occlusion and reperfusion [35]. Cardiac myocyte death progresses via apoptosis and necrosis. Apoptosis is a highly regulated form of programmed cell death characterized by morphological features including the formation of membrane blebs, nuclear condensation, and deoxyribonucleic acid (DNA) fragmentation, as well as the activation of caspase enzymes [36]. In contrast, necrosis is considered to be an unregulated form of cell death characterized by the formation of cellular debris and an ensuing inflammatory response [36]. Both apoptosis and necrosis contribute to cardiac myocyte death and infarction following ischemia and reperfusion, where cardiac myocyte death likely represents a spectrum of both apoptosis and necrosis depending on the severity of IR injury [37].

Morphologically, myocardial infarcts progress as a wavefront of cardiac myocyte death from the ischemic subendocardium to the subepicardium [2]. As infarcted tissue is no longer capable of contraction, global heart function may be impaired. Following AMI, ventricular dysfunction is accompanied by reactive hypertrophy to compensate for cardiac myocyte loss. However, hypertrophy can become maladaptive and other adverse remodeling processes may ensue including infarct expansion, left ventricular (LV) dilation, and interstitial fibrosis which may progress and ultimately result in heart failure and a spectrum of ischemic heart diseases as well as death [35,38].

1.3 Non-Pharmacological and Pharmacological Approaches to the Treatment of Ischemic Heart Disease

The treatment of ischemic heart disease has centered on altering O_2 use, either by increasing O_2 availability or by decreasing O_2 demand. This can be achieved via hypothermic cardioplegic arrest, an established method of tissue preservation *ex vivo* which depresses cardiac O_2 demand, or through the use of pharmacological agents that alter systemic and cardiac hemodynamics, as well as cardiac contractility. Pharmacological mainstays in the management of ischemic heart disease include angiotensin converting enzyme (ACE) inhibitors, L-type Ca^{2+} channel blockers, nitrates, and β-adrenoceptor antagonists. While ACE inhibitors, L-type Ca^{2+} channel blockers, and nitrates improve the hemodynamic profile by decreasing both preload and systemic vascular resistance via vasodilatation thereby increasing O_2 supply and decreasing O_2 demand, β-adrenoceptor antagonists exert their anti-ischemic effects via a negative chronotropic and inotropic action, thereby reducing O_2 demand. In addition recent evidence suggests that β-adrenoceptor antagonists also alter energy substrate metabolism, specifically they appear to shift substrate preference from free fatty acids (FFAs) towards glucose utilization [39]. Recently several pharmacological agents, including perhexeline, trimetazidine, and etomoxir have received renewed interest as anti-ischemic agents. These compounds appear to act by mechanisms independent of alterations in hemodynamics and contractility, but rather by mechanisms related to their ability to induce a shift in energy substrate metabolism from FFAs towards glucose, which may lead to O_2 sparing effects, as well as reduced intracellular acidosis and Ca^{2+} overload, effects that are beneficial in the treatment of ischemic heart disease [40].

Glucose Metabolism and Utilization

1.4 Glucose Uptake

The cellular uptake of glucose is a complex process coupled between the rate of glucose delivery to the interstitial space, the rate of glucose transport into cells, and the rate at which glucose is phosphorylated [41]. Although the particulars by which glucose moves across capillaries is not known, the mechanism is generally regarded to be that of passive diffusion, where glucose moves down its chemical gradient across the capillaries and into the interstitial space [42]. As glucose is a hydrophilic molecule and its rate of movement across the lipid bilayer exceeds that which can be explained by passive diffusion, glucose enters the cell via facilitative transport. The cellular transport of glucose is mediated by a family of glucose transporters (GLUTs), of which there are currently twelve identified isoforms [43]. Of particular relevance to the cardiac myocyte are GLUT 1 and GLUT 4. The majority of GLUT 4 is stored in the intracellular compartment, whereas GLUT 1 has a pronounced sarcolemmal localization [44]. The stimulation of myocardial glucose transport involves an increase in the recruitment of GLUT 1 as well as GLUT 4 from intracellular compartments to the sarcolemma [45].

Following transport, glucose is phosphorylated by hexokinase I and hexokinase II forming glucose-6-phosphate (G-6-P). Hexokinase I predominates in the fetal newborn heart, whereas hexokinase II predominates in the adult heart [46]. As glucose is phosphorylated nearly as rapidly as it is translocated into the cell, the intracellular concentration of free glucose is negligible, thereby maintaining a steep downward concentration gradient from the interstitial- to intracellular-space [42]. G-6-P is effectively

trapped in the cell is a substrate for either of two metabolic fates, storage in the form of glycogen, or catabolism by glycolysis.

1.5 Glycogen Metabolism

Although a large proportion of G-6-P enters the glycolytic pathway, it is also a substrate for the synthesis of glycogen, the intracellular storage glucan for excess glucose [3]. Glycogen metabolism is a dynamic process, consisting of simultaneous synthesis and degradation. For G-6-P to enter into the synthetic pathway for glycogen, it must first be converted to glucose-1-phosphate (G-1-P), by the action of phosphoglucomutase [9].

The enzymes involved in glycogen synthesis are uridine diphosphate (UDP)-glucose phosphorylase, glycogen synthase, and glycogen branching enzyme. UDP-glucose phosphorylase catalyzes the reaction between G-1-P and uridine triphosphate (UTP), forming UDP-glucose with the release and subsequent hydrolysis of pyrophosphate (PPi) [47]. The glycogen synthase reaction transfers the glucose moiety of UDP-glucose to a previously present glycogen chain via the formation of $\alpha(1\rightarrow4)$ linkages, yielding α-amylose [47,48]. Branching to form glycogen is accomplished via glycogen branching enzyme ([1,4→1,6])-transglycosylase), which transfers a seven-residue terminal segment from an $\alpha(1\rightarrow4)$ linked glucan to the carbon-6 (C6)-OH on a similar or separate $\alpha(1\rightarrow4)$ terminal chain [9,47].

The enzymes involved in glycogen degradation or glycogenolysis are glycogen phosphorylase, glycogen debranching enzyme, and phosphoglucomutase [49]. Glycogen phosphorylase catalyzes the phosphorolysis of glycogen (i.e. bond cleavage by the substitution of a phosphate group) yielding G-1-P. This reaction proceeds only if the terminal glucosyl unit is at least five units away from a branch point. Glycogen

debranching enzyme (an [1,4]-transglycosylase) transfers α(1→4) linked trisaccharide to the non-reducing end of another branch, forming a new α(1→4) linkage with three more units available for phosphorylysis [50]. The remaining α(1→6) bond linking the remaining glucosyl unit to the main glycogen chain is hydrolyzed (not phosphorylyzed) to yield glucose and debranched glycogen [9]. G-1-P liberated by the glycogen phosphorylase reaction is converted to G-6-P by the action of phosphoglucomutase and can either be recycled into glycogen or enter the glycolytic pathway.

1.5.1 Regulation of Glycogen Metabolism

The dynamic nature of glycogen metabolism is tightly regulated via allosteric control as well as hormonal control due to covalent modifications of the enzymes involved in glycogen metabolism [51]. Both glycogen synthase and glycogen phosphorylase are under allosteric control by effectors including ATP, adenosine monophosphate (AMP), and G-6-P [52,53]. Glycogen synthase is activated by G-6-P [52], whereas glycogen phosphorylase is activated by AMP and inhibited by ATP and G-6-P [51,53]. This suggests that when the concentration of ATP and G-6-P is high, glycogen synthesis is favored, while glycogenolysis is inhibited. In contrast, when the concentration of ATP and G-6-P is low and the concentration of AMP is elevated, glycogenolysis is favored, while glycogen synthesis is inhibited. The allosteric control of glycogen metabolism is also coupled tightly to control via phosphorylation and dephosphorylation. The covalent modification of glycogen synthase and glycogen phosphorylase occurs due to the interconversion of each enzyme between two states, active (*a*) and inactive (*b*) [9]. Whereas phosphorylation inactivates glycogen synthase (*b*) and activates glycogen phosphorylase (*a*), dephosphorylation activates glycogen synthase

(*a*) and inactivates glycogen phosphorylase (*b*) [9,48]. The phosphorylation of both glycogen synthase and glycogen phosphorylase is mediated by phosphorylase kinase as well as protein kinse A (PKA), while dephosphorylation is mediated by phosphoprotein phosphatase-1 [9,48]. Ultimately whether glycogen accumulates or is degraded depends on the balance of the relative activities of glycogen synthase and glycogen phosphorylase, as well as the availability of substrate.

1.6 Glycolysis

Glycolysis is the biochemical process where by glucose is converted to lactate or pyruvate in the absence or presence of O_2, respectively [3]. The metabolism of glucose by the glycolytic pathway occurs in the cytosol, where the enzymes involved in glycolysis are located [9]. The reactions involved in glycolysis can be divided into two stages. Stage 1 or the preparatory stage (reactions 1-5), where glucose is phosphorylated and cleaved to yield 2 molecules of glyceraldehyde-3-phosphate, utilizes 2 molecules of ATP [49]. Stage 2 (reactions 6-10), where 2 molecules of glyceraldehyde-3-phosphate are converted to pyruvate, generates 4 molecules of ATP [49]. Thus there is a net production of 2 moles (mol) ATP/ 1 mol of exogenous glucose that passes through glycolysis. As endogenous glucose, which is liberated from the glycogen pool as G-1-P, and subsequently converted to G-6-P through the action of phosphoglucomutase does not consume ATP as it bypasses the hexokinase reaction, there is a net generation of 3 mol ATP/1 mol endogenous glucose [51].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) the first enzyme of the ATP generating stage of glycolysis is involved in the oxidation and phosphorylation of glyceraldehyde phosphate coupled to the production of nicotinamide adenine dinucleotide

(reduced form) (NADH) from nicotinamide adenine dinucleotide (NAD^+) [51]. Thus to ensure flux through GAPDH is not restricted NADH must be continually reoxidized to NAD^+. This is accomplished by one of two routes. In anaerobic conditions in the absence of O_2, NADH is reoxidized by the enzyme lactate dehydrogenase (LDH), which converts pyruvate to lactate [51]. In the presence of oxygen, under aerobic conditions NADH is reoxidized by the mitochondrial electron transport chain [9].

1.6.1 Regulation of Glycolysis

The enzyme 6-phosphofructo-1-kinase (PFK-1) is the first regulatory site that commits glucose to catabolism by glycolysis by catalyzing the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate [54]. PFK-1 is regulated primarily by allosteric mechanisms. The activity of PFK-1 is allosterically activated by AMP and fructose-2,6-bisphosphate [51]. Fructose-2,6-bisphosphate is a potent stimulator of glycolysis and itself is formed from fructose-6-phosphate by the action of 6-phosphofructo-2-kinase (PFK-2), whose activity increases in response to several of the effectors of the PFK-1 reaction including AMP and inorganic phosphate [55,56]. Flux through PFK-1 is allosterically inhibited by ATP, citrate, and H^+ [54].

Pyruvate kinase catalyses the conversion of phosphoenolpyruvate to pyruvate with the concomitant formation of ATP in the terminal step of glycolysis [57]. The enzyme may contribute to the regulation of glycolysis as the product of PFK-1, fructose-1,6-phosphate increases the affinity pyruvate kinase for its substrate, phosphoenolpyruvate [57]. Such a feed forward mechanism would allow PFK-1 to accelerate glycolysis, while avoiding the accumulation of glycolytic intermediates.

1.7 Glucose Oxidation

Under aerobic conditions pyruvate is the end product of glycolysis. The aerobic disposal of pyruvate (glucose oxidation) requires that it be transported into mitochondria via a monocarboxylate carrier [58]. Once in the mitochondrial matrix, the majority of pyruvate undergoes oxidative decarboxylation by the pyruvate dehydrogenase complex (PDC) to yield acetyl-coenzyme A (acetyl-CoA), which is then fed into the tricarboxylic acid (TCA) cycle, where the acetyl groups undergo complete oxidation liberating carbon dioxide (CO_2) [59].

1.7.1 Regulation of Glucose Oxidation

PDC is a mitochondrial multi-enyme complex consisting of pyruvate dehydrogenase (PDH), PDH kinase, and PDH phosphatase, the complex is regulated by its substrates and products as well as by covalent modification [60,61]. Normally only a small portion (~20%) of PDH is in the active form, this percentage is increased in response to an increase in glycolytic flux, in the face of an increase in workload or in the presence of catecholamines [62]. PDH is also sensitive to inhibition by its products as increased ratios of $NADH/NAD^+$ and acetyl-CoA/CoA decrease the rate of pyruvate decarboxylation [62]. With regards to covalent modification, PDH phosphatase dephosphorylates and activates PDH. PDH kinase, in response to acetyl-CoA and NADH (produced primarily from fatty acid β-oxidation) phosphorylates and inhibits PDH, and thus restricts the oxidation of carbon units derived from glycolysis [60,61].

Fatty Acid Metabolism and Utilization

1.8 Fatty Acid Uptake and Activation

As a fuel source, fat constitutes the body's major fuel reserve. A prerequisite for the catabolism of fat is the liberation of non-esterified free fatty acids (NEFA) from stored or ingested triacylglycerol via the action of either hormone sensitive lipase or lipoprotein lipase respectively [3,9]. In the heart and skeletal muscle intracellular triacylglycerol represents a significant source of NEFA. Although NEFA can be liberated from circulating chylomicrons and very low density lipoprotein (VLDL), circulating NEFA bound to albumin are the main extra-cellular substrate for mitochondrial β-oxidation.

The uptake of NEFA is governed by the NEFA concentration gradient across the sarcolemmal membrane. The cellular uptake of NEFA has long been considered to be a passive mechanism, however, it's saturable nature and sensitivity to competitive inhibition in isolated cardiac myocytes implicates a protein component [63,64]. The putative protein mediators implicated in the uptake of NEFA are the plasma membrane fatty acid binding protein (FABPpm), fatty acid translocase/CD36 (FAT/CD36), and members of the bifunctional family of fatty acid transport proteins (FATPs), which exhibit intrinsic acyl-CoA synthetase activity. Following dissociation from plasma albumin, NEFA can either directly enter the cell by the process of passive diffusion directly, or indirectly following binding to FABPpm. Conversely, NEFA can enter cells by the process of facilitated transport being translocated either directly following dissociation from albumin by FATPs or FAT/CD36, or following binding to FABPpm and subsequent translocation by FAT/CD36 [44].

Once NEFA have gained entry to the cytosol, they require activation prior to further metabolism. NEFA are activated through the formation of fatty acyl-CoA moieties through an ATP and CoASH dependent process catalyzed by a family of acyl-CoA synthetases. Recent evidence suggests that NEFA transported by FATP family members may be directly activated as these proteins possess acyl-CoA synthetase activity [65]. In contrast, NEFA which enter the cell by passive diffusion or translocation by FAT/CD36 are activated by cytosolic acyl-CoA synthetases. In the cytosol fatty acyl-CoA moieties are bound to acyl-CoA binding protein (ACBP), and can be utilized for a variety of purposes including phospholipid and triglyceride synthesis, signal transduction, or oxidation for energy production.

1.9 Mitochondrial Fatty Acid Uptake and Oxidation

As the inner mitochondrial membrane is impermeable to fatty acyl-CoA molecules, the entry of fatty acyl-CoAs into the mitochondrial matrix is regulated by a complex of proteins using carnitine as a shuttle mechanism. Carnitine palmitoyl transferase I (CPT-I), localized to the outer mitochondrial membrane converts the fatty acyl-CoA into an acyl-carnitine [66], which is subsequently translocated into the mitochondrial matrix by carnitine translocase, and re-converted to a fatty acyl-CoA moiety by carnitine palmitoyl transferase II (CPT-II) located on the internal leaflet of the inner mitochondrial membrane [67,68]. The degradation of fatty acyl-CoA molecules occurs in the mitochondrial matrix.

The catabolism of fatty acyl-CoA molecules proceeds through the β-oxidation spiral catalyzed by the enzymes acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-L-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase. β-oxidation

progressively dismembers fatty acyl-CoA molecules by liberating acetyl-CoA (2 carbon units) for further metabolism by the TCA cycle, and producing reducing equivalents in the form of NADH and reduced flavin adenine dinucleotide (FADH) for subsequent oxidation by the electron transport chain [67,68].

1.9.1 Regulation of Fatty Acid Oxidation

Important factors regulating the rate of fatty acid oxidation are the level of circulating NEFA in the plasma and the intracellular level of malonyl-CoA [69]. The concentration of NEFA in the plasma is determined by prandial state as well as hormonal state. Plasma NEFA concentration increases with fasting, and decreases in the fed state due to the anabolic and anti-lipolytic effects of insulin [3]. An increase in catecholamine discharge also increases circulating NEFA concentration by increasing adipose tissue lipolysis [3]. An increase in the delivery of fatty acids to the site of utilization can increase the rate of fatty acid oxidation.

Malonyl-CoA is an endogenous regulator of fatty acid β-oxidation. The intracellular levels of malonyl-CoA are determined by energy demand and its rates of synthesis and degradation [69]. Mitochondrial acetyl-CoA via the formation of acetylcarnitine can be transported into the cytosol by the formation of acetylcarnitine, and subsequently be reconverted to cytosolic acetyl-CoA by the action of cyotsolic carnitine acetyl transferase [67]. Malonyl-CoA is synthesized from cytosolic acetyl-CoA through the enzyme acetyl-CoA carboxylase (ACC), while being degraded by the enzyme malonyl-CoA decarboxylase (MCD) [69]. Malonyl-CoA regulates fatty acid β-oxidation by inhibiting the activity of CPT-1, the rate limiting enzyme of mitochondrial fatty acid

uptake, thereby controlling the entry of fatty acids into the mitochondria and subsequent oxidation and production of acetyl-CoA for entry into the TCA cycle [70].

1.10 The Tricarboxylic Acid Cycle

The TCA cycle is a series of eight enzymatic reactions occurring in the mitochondrial matrix. The TCA cycle oxidizes acetyl-CoA derived from both glucose and fatty acid metabolism in a cumulative manner liberating CO_2 and concomitantly generating reducing equivalents in the form of NADH and FADH [49]. The reducing equivalents are subsequently fed into the mitochondrial electron transport chain for use in the production of ATP production via oxidative phosphorylation [49].

1.10.1 Regulation of the Tricarboxylic Acid Cycle

Regulation of the TCA cycle appears to be exerted primarily by the $NADH/NAD^+$ ratio. Increases in ATP demand drive oxidative phosphorylation, and so decreases the ratio of $NADH/NAD^+$ [71]. This in turn stimulates the activity of several enzymes of the TCA cycle including isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and malate dehydrogenase thereby increasing TCA cycle activity. Increases in the intramitochondrial Ca^{2+} concentration also increase TCA cycle activity as both isocitrate dehydrogenase and α-ketoglutarate dehydrogenase are sensitive to Ca^{2+} [72].

Integrative Regulation of Myocardial Energy Substrate Metabolism

1.11 Substrate Competition – The Glucose Fatty Acid Cycle

Under normal, physiological conditions the metabolic fuels involved in sustaining cardiac function are fatty acids and carbohydrates (mainly glucose and lactate). Fatty acids provide the major source of oxidative substrate for cardiac energy metabolism,

accounting for 60-80% of oxygen consumption, with a much lesser contribution from glucose and lactate [69]. This preference is likely due to the higher ATP yield obtained from the oxidation of fatty acids compared to the oxidation of glucose (e.g. 105 ATP/palmitate molecule vs. 30 ATP/glucose molecule). However, preference for fatty acids as an oxidative fuel at the expense of glucose also carries disadvantages, attributed to the greater amount of O_2 required per ATP produced. The competition for oxidative metabolism between fatty acids and glucose was originally described by Randle et al. in 1963 as the glucose fatty acid cycle [73].

The metabolic relationship between fatty acid and glucose metabolism is reciprocal. The provision of glucose promotes glucose oxidation, as well as glucose and lipid storage; whereas, provision of NEFA promotes fatty acid oxidation and storage, while inhibiting glucose oxidation [74]. The molecular mechanisms underlying this reciprocal relationship are manifest at multiple levels of the pathways involved in the catabolism of glucose. Acetyl-CoA produced from the β-oxidation of fatty acids inhibits the PDC, which in turn can lead to an inhibition of PFK-1 by citrate and of hexokinase by G-6-P [74]. The reciprocal regulation of glucose metabolism by fatty acid oxidation occurs in a hierarchical manner, with glucose oxidation being inhibited to the largest extent, followed by a lesser effect on glycolysis and glucose uptake. The effects of fatty acid β-oxidation-induced inhibition of glucose metabolism are manifest primarily as an uncoupling between glycolysis and glucose oxidation. Thus H^+ produced from the hydrolysis of glycolytically derived ATP are not consumed by the mitochondrial electron transport chain, and have the potential to produce intracellular acidification, especially

during periods of ischemia where blood flow is insufficient to remove metabolic by-products.

1.12 Myocardial Energy Substrate Metabolism During Ischemia and Reperfusion

The rate of ATP turnover in the heart is very rapid, with the myocardial ATP pool turning over approximately every 12 seconds due to a high ATP demand in order to maintain contractile function (60% of total ATP demand) and ionic homeostasis (40% of ATP demand) [75]. As the major effect of myocardial ischemia is the inhibition of oxidative ATP production in the mitochondria from the catabolism of pyruvate and fatty acids, both contractile function and ionic homeostasis can be compromised as a result of ischemia.

Due to its ability to generate ATP in the absence of O_2, glycolysis becomes increasingly important during periods of ischemia. During periods of mild to moderate ischemia, flux through glycolysis is stimulated/or leads to an increase in glucose uptake and increased glycogen mobilization, in response to a fall in cellular citrate levels, and stimulation of PFK-1 [54]. As glycolysis accounts for the majority of myocardial glucose use, its relative rate may dictate total, overall rates of glucose uptake. Although glycolysis can provide ATP in order to correct and maintain ionic homeostasis during ischemia, the hydrolysis of glycolytically derived ATP in the absence of subsequent pyruvate oxidation leads to an accumulation of lactate and H^+, which can further aggravate ionic disturbances brought about by ischemia. Thus during periods of ischemia when glycolysis is accelerated a greater proportion of ATP hydrolysis must be diverted towards performing chemical work (re-establishing ionic balance) than contractile work [55]. In situations of severe ischemia, where substrate supply is limiting, the myocardial

glycogen pool becomes the primary source of glucose for glycolysis. However, in the absence of sufficient flow, the metabolic by-products of anaerobic glycolysis are not removed, and flux through the pathway is eventually inhibited by the effects of acidosis primarily at the levels of PFK-1 and GAPDH [51,54].

In the post-ischemic period during reperfusion, the rates of oxidative fatty acid metabolism recovery rapidly to pre-ischemic values at the expense of glucose oxidation, while contractile function is depressed [76,77]. This rapid recovery of fatty acid oxidation can contribute to an ongoing uncoupling of glucose metabolism thus causing intracellular acidosis, and impairing the recovery of cardiac function and efficiency despite the restoration of flow [78]. Intracellular acidosis impairs the response of the contractile filaments to Ca^{2+}, thereby contributing the impaired recovery of function during reperfusion. Furthermore, as extracellular pH is quickly normalizes upon reperfusion, there is large pH gradient across the sarcolemmal membrane that promotes Na^{+}/H^{+} exchange, increasing intracellular Na^{+}, which in turn promotes Na^{+}/Ca^{2+} exchange, and the sequelae associated with intracellular Ca^{2+} overload including myocardial stunning and myocyte cell death described in detail above.

1.13 Targeting Myocardial Energy Substrate Metabolism to Limit Ischemia-Reperfusion Injury

Optimizing energy substrate metabolism in the ischemic and reperfused myocardium may represent a novel mechanism to limit IR injury and enhance the recovery of mechanical function during the post-ischemic period, whether the ischemic insult in question is the result of some underlying pathophysiology or due to elective surgical procedures. Pharmacological agents that shift the balance between fatty acid and

glucose utilization towards glucose utilization have received considerable attention. In particular pharmacological agents that improve the coupling between glycolysis and glucose oxidation, either by inhibiting fatty acid β-oxidation and/or by stimulating glucose oxidation show promise as potential cardioprotective interventions. The compound trimetazidine partially inhibits fatty acid β-oxidation due to its inhibitory effects on the mitochondrial enzyme 3-ketoacyl-CoA thiolase, and is clinically approved for the treatment of angina in Europe [79,80]. Dichloroacetate exerts its cardioprotective effects by stimulating flux through the PDC by inhibiting PDH kinase thereby preventing inhibition of PDH by phosphorylation [75].

1.14 Adenosine – Effects on Glucose Metabolism and Myocardial Ischemia-Reperfusion Injury

The purine nucleoside, adenosine has been demonstrated to influence glucose uptake and intermediary carbohydrate metabolism in a variety of experimental systems. In adipocytes and erythrocytes, adenosine enhances glucose metabolism via increased glucose uptake [81], glycolysis [82], and glucose oxidation [83]. However, less well characterized is the role of adenosine in regulating cardiac glucose metabolism. Previous reports utilizing isolated rat hearts perfused with glucose as the sole energy substrate demonstrate adenosine-induced increases in glucose uptake [84,85] and glycolysis [86,87], and suggest that these alterations in glucose metabolism in part contribute the ability of adenosine to attenuate myocardial IR injury. In contrast, adenosine partially inhibits glycolysis in isolated rat hearts perfused with both glucose and fatty acids as energy substrates [88], an effect also implicated in its ability to attenuate IR injury [89].

1.14.1 Adenosine and Glucose Metabolism in the Fatty Acid Perfused Heart

As there is an absolute requirement for O_2 for the aerobic disposal of pyruvate (glucose oxidation), an alternative mechanism to prevent the uncoupling of glucose metabolism during and following ischemia when both coronary flow and O_2 are re-introduced is to limit the rate of glycolysis. Adenosine decreases glycolysis and improves the coupling between glycolysis and glucose oxidation, thereby inhibiting H^+ production in the fatty acid perfused heart [88]. Numerous experimental models also demonstrate the marked cardioprotective effects of adenosine ranging from a reduction in the incidence of arrhythmias and myocardial stunning, to a reduction in infarct size (i.e. protection against both reversible and irreversible myocardial injury) [90]. Furthermore, the metabolic actions of adenosine are intimately linked with its effects on the recovery of function following myocardial IR. In this regard, the adenosine-induced inhibition of glycolysis is associated with a significant improvement in the recovery of LV work following IR [89]. Interestingly, adenosine-induced cardioprotection appears to be associated with the effects of adenosine on glucose metabolism, as an adenosine-induced stimulation of glycolysis and H^+ production following transient antecedent ischemia impairs the recovery of LV work following severe ischemia [91]. Although the effects of adenosine on myocardial carbohydrate metabolism and post-ischemic function in isolated rat hearts perfused with glucose and fatty acids are related, little is known about the biochemical/cellular signaling molecules involved in the transduction of these effects.

Protein Kinases and Myocardial Ischemia-Reperfusion Injury

1.15 Mitogen Activated Protein Kinases

One of the major systems involved in signal transduction from the plasma membrane to intracellular targets is the highly conserved mitogen activated protein kinase (MAPK) superfamily. The three MAPK families identified in the myocardium are the extracellular signal-regulated protein kinases (ERKs), and the two stress activated protein kinases (SAPKs), c-jun N-terminal protein kinases (JNKs) and p38 MAPK. MAPK signaling cascades are organized into three-tiered modules of protein phosphorylation and activation, where by activation of MAPK kinase kinases (MAPKKKs) results in the phosphorylation and activation of MAPK kinases (MEK), which are dual specificity kinases that phosphorylate and activate a MAPK [92].

ERK 1/2 are primarily activated in response to mitogenic/growth factor stimulation. MEK 1/2 is the immediate upstream regulator of ERK 1/2, and activates ERK 1/2 by phosphorylation of a threonine-glutamate-tyrosine motif with in its activation loop [92]. The JNKs are activated in response to cellular stresses including ultraviolet (UV) irradiation, and phosphorylate the transcription factor c-Jun within its N-terminal activation domain. The JNKs are activated MEK 4 and MEK7 via phosphorylation of a threonine-proline-tyrosine motif [93]. The p38 MAPKs are primarily activated by cellular stresses including osmotic shock, heat stress, metabolic inhibition, and ATP depletion [94]. MEK 3/6 activate p38 MAPK via phosphorylation of a threonine-glycine-tyrosine motif. All three MAPK family members have been identified in the heart, and may play roles in various cardiac pathophysiological states ranging from cardiac hypertrophy, heart failure, and myocardial IR injury [92].

1.15.1 Mitogen Activated Protein Kinases and Myocardial Ischemia-Reperfusion Injury

Activation of the ERK cascade has been demonstrated following myocardial IR in various experimental models both *in vivo* and *in vitro* [95,96,97]. It is generally accepted that activation of the ERK cascade during reperfusion is cardioprotective, as selective inhibition of ERK1/2 exacerbates cardiac myocyte apoptosis and IR injury [97]. In addition, cardioprotection induced by ischemic preconditioning (IPC), where by brief sub-lethal periods of ischemia protect the heart from a subsequent, and otherwise lethal period of ischemia requires the phosphorylation of ERK1/2 during reperfusion [98]. The activation of the ERK cascade is also implicated in the cardioprotective phenomenon of post-conditioning, where reflow following otherwise lethal ischemia is restored in an intermittent manner as opposed to abrupt reperfusion [99].

The role of JNKs in myocardial IR injury appears to be controversial, with studies suggesting that the activation of the JNK cascade is both protective and deleterious. With regards to cardioprotection, activation of the JNKs with okadaic acid correlates with a reduction in infarct size in the porcine heart *in vivo* [100]. Furthermore, increased JNK phosphorylation is also associated with the reduction of infarct size in response to IPC in the porcine heart *in vivo* [101]. These reductions in infarct size may be related to the ability of activated JNKs to decrease apoptosis following myocardial IR [102,103].

In contrast to the above reports, there is also evidence that activation of the JNK cascade is detrimental in myocardial IR. Increased activity of the JNKs has been documented in the failing human heart, suggesting that the JNK cascade may be important in cardiac pathophysiology [104]. ROS, which are produced following IR also

contribute to the activation of the JNK cascade [105,106]. Furthermore, inhibition of the JNK cascade following myocardial IR is cardioprotective in the *in vivo* rat model by virtue of decreasing apoptosis and infarct size [107].

The role of p38 MAPK in the evolution of myocardial IR injury is also controversial, with studies suggesting protective as well as detrimental consequences. Evidence suggesting that the activation of p38 MAPK is cardioprotective comes from studies of IPC. Several studies demonstrate that p38 MAPK phosphorylation and activity are increased during the sustained period of ischemia in preconditioned hearts, and that inhibitors of p38 MAPK administered prior to the onset of sustained ischemia abolish the protective effects of IPC [108,109]. Furthermore, a recent report indicates that cardiac specific overexpression of MEK6, the immediate upstream kinase for p38 MAPK confers improved functional recovery following IR *in vitro* [110].

In contrast, experimental evidence exists that demonstrates that inhibition of p38 MAPK following myocardial IR is cardioprotective. p38 MAPK is activated by ROS in cultured cardiac myocytes, and its inhibition protects from ROS-induced apoptotic cell death [111]. Moreover, in isolated perfused heart models p38 MAPK is activated following IR, and inhibition of p38 MAPK is a cardioprotective intervention as assessed by an attenuation of apoptosis and necrosis, and improved recovery of post-ischemic contractility as well as cardiac function [112,113]. The cardioprotective effects of p38 MAPK inhibition are also extended to *in vivo* models of IR, where p38 MAPK inhibition prevents neutrophil accumulation, attenuates apoptosis and infarct size, as well as functional detriment [114,115,116]. Although p38 MAPK has been demonstrated to participate in glucose uptake in the ischemic myocardium, the effects of p38 MAPK on cardiac

energy substrate metabolism and its relation to cardioprotection have not yet been characterized.

1.16 5'-AMP-Activated Protein Kinase

5'-AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine protein kinase comprised of α, β, and γ subunits, each of which has two or more isoforms and are the products of distinct genes [117]. The α subunit contains the catalytic domain, and an important regulatory residue, threonine-172, which is phosphorylated by upstream kinases [118]. The β subunit contains a glycogen-binding domain [118]. The γ subunit contains 4 cystathionine β-synthase domains that function in pairs to bind a single molecule of ATP or AMP in a mutually exclusive manner [118]. The AMPK heterotrimer is organized such that the α subunit binds to both the β and γ subunits, however, there is no direct interaction between the β and γ subunits [119]. AMPK is activated by an increase in AMP concentration and phosphorylation by upstream AMPK kinases (AMPKKs) in response to a wide variety of cellular stresses including ischemia, and hypoxia that deplete cellular ATP [120]. The binding of AMP to the γ subunit of AMPK induces a conformational change and promotes the activation of AMPK by several mechanisms: (i) it allosterically activates the catalytic α subunit, (ii) it promotes phosphorylation of the regulatory threonine-172 residue, which is required for maximal enzyme activity, and (iii) it renders AMPK a less suitable substrate for dephosphorylation by protein phosphatase 2C [121].

Due to its sensitivity to AMP and ATP, AMPK has been termed a cellular energy sensor [120]. As such, the activation of AMPK is generally associated with the coordinated stimulation of catabolic ATP producing processes, and inhibition of anabolic ATP

consuming processes. This is achieved through the AMPK-mediated phosphorylation of target proteins involved in the regulation of cellular energy substrate metabolism. The activation of AMPK is reported to increase the phosphorylation of acetyl-CoA carboxylase resulting in its inhibition and subsequent acceleration of fatty acid β-oxidation (described above) [121]. AMPK is also reported to phosphorylate and increase the activity of PFK-2, which generates fructose-2,6-bisphosphate, a potent stimulator of glycolysis, via stimulation of the enzyme PFK-1 [122]. Conversely, AMPK is reported to phosphorylate and inhibit glycogen synthase, and thus the storage of glucose as glycogen [118].

However, several recently identified mutations in the regulatory γ subunit of AMPK, including arginine 300 to glutamine in the γ_3 isoform, as well as asparagine 488 to isoleucine, arginine 302 to glutamine, and arginine 531 to glycine in the γ_2 isoform suggest that the interaction between AMPK and glycogen metabolism is very complex [123]. Of particular interest, hearts from transgenic mice expressing the arginine 302 to glutamine or arginine 531 to glycine mutations exhibit marked cardiac hypertrophy associated with a loss of function of AMPK and excessive myocardial glycogen accumulation [124, 125], whereas hearts from transgenic mice expressing the asparagine 488 to isoleucine mutation exhibit cardiac hypertrophy associated with a gain of function of AMPK and excessive myocardial glycogen accumulation [126]. Thus the relationship(s) between AMPK and cardiac glycogen metabolism require further clarification. Furthermore, whether the effects of AMPK on the pathways of cellular energy metabolism confer cardioprotection following IR is the topic of intense investigation.

1.16.1 5'-AMP-Activated Protein Kinase and Myocardial Ischemia-Reperfusion Injury

Recent experimental evidence suggests that the activation of AMPK may act to limit IR injury. AMPK has been shown to participate in IPC of the liver through the use of the AMPK activator, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) [127], as well as cardiac myocyte IPC [128]. Furthermore, AMPK activation also appears to impart anti-inflammatory properties of reduced leukocyte rolling and adhesion to jejunal endothelium, and so may have the potential to prevent the phenomenon of no reflow in myocardial IR [129]. The cardioprotective effects of adiponectin *in vivo* manifest as an inhibition of cardiac myocyte apoptosis and reduction in infarct size following IR are associated with increased AMPK phosphorylation, indicative of increased AMPK activity [130]. Conversely, the inhibition of AMPK in transgenic mice expressing a kinase dead mutant of AMPK is associated with increased apoptosis, lower ATP content, and a failure to augment fatty-acid oxidation during low-flow ischemia, suggesting that AMPK indeed play a protective role during/following myocardial IR [131].

The above study suggests that energy metabolism is an important component of cardioprotection induced by the activation of AMPK following IR. However, in contrast to this is the observation that elevated AMPK activity accompanies accelerated rates of fatty acid oxidation in the post-ischemic period [132,133]. These accelerated rates of fatty acid oxidation may contribute to the poor recovery contractile function during reperfusion in part by uncoupling glycolysis and glucose oxidation, and thus increasing H^+ production; however, evidence for the activation of AMPK and the uncoupling of glycolysis and glucose oxidation in aerobically perfused hearts is lacking.

Stress-Responsive Protein Kinases

1.17 AMPK and p38 MAPK Signaling and the Regulation of Glucose Metabolism

The stress-responsive protein kinases, AMPK and p38 MAPK share several common activating stimuli including muscle contraction, hypoxia, and ATP depletion, suggesting that the two kinases may participate in a common signaling pathway [134]. However, the overall contribution of both AMPK and p38 MAPK to the regulation of myocardial IR injury and its inter-relationship with energy substrate metabolism remains to be characterized. Previous reports implicate AMPK and p38 MAPK in the regulation of apoptosis in both cardiac fibroblasts as well as isolated cardiac myocytes, and place AMPK upstream of p38 MAPK, suggesting that AMPK and p38 MAPK may regulate IR injury in a coordinated manner [135,136].

Important determinants of the extent of myocardial IR injury are the alterations in energy substrate metabolism that occur both during and following ischemia. This is exemplified by the ability of trimetazidine and dichloroacetate to stimulate glucose oxidation, and of adenosine to improve the coupling between glycolysis and glucose oxidation and enhance the recovery of post-ischemic function following IR [78,79], as well as the ability of adenosine to uncouple glycolysis and glucose oxidation, and impair the recovery of post-ischemic function following IR in hearts stressed by transient ischemia [91]. With regards to fatty acid and carbohydrate metabolism, AMPK and p38 MAPK have been implicated in the regulation of fatty acid β-oxidation, and glucose uptake, which determines the availability of substrate for glycolysis and glycogen metabolism in a variety of systems ranging from C2C12 skeletal muscle myoblasts, and isolated cardiac myocytes [137,138].

The contribution of AMPK and p38 MAPK, and their interaction in the regulation of energy metabolism and IR injury in the isolated, fatty acid perfused rat heart remains to be characterized. The recognition that stress-responsive protein kinases control both energy metabolism and IR injury may provide novel and viable pharmacological targets to limit the impact of myocardial IR injury.

Hypothesis and Objectives

1.18 General Hypothesis

Alterations in cardiac energy substrate metabolism that occur during and following ischemia are important determinants of the severity of myocardial ischemia reperfusion injury, as well as the degree of functional recovery. The emergence of the stress-responsive protein kinases, AMPK and p38 MAPK as regulators of carbohydrate and fatty acid metabolism, may represent novel pharmacological targets to ameliorate or limit IR injury in part due to their effects on the pathways of energy substrate utilization and metabolism.

1.18.1 Specific Hypotheses

The specific hypotheses of this thesis are outlined and described within the individual chapters pertaining to experimental results. The hypotheses addressed are listed below.

1. Hypothesis – Chapter 2:

Inhibition of MAPKs (ERK 1/2 or p38 MAPK) during hypothermic ischemia and reperfusion, respectively is cardioprotective, and thus enhances the recovery of post-ischemic function.

2. Hypothesis – Chapter 3:

The activation of AMPK is responsible for the adenosine-induced stimulation of glycolysis and H^+ production during aerobic perfusion, in hearts stressed by transient ischemia. Furthermore, that the activation of AMPK stimulates fatty acid oxidation, glucose uptake, and glycogenolysis, similar to the activation of AMPK in response to ischemia.

3. Hypothesis – Chapter 4:

AMPK and p38 MAPK form a functional signaling cascade to regulate myocardial glycolysis and glycogen metabolism following adenosine treatment in hearts stressed by transient ischemia.

4. Hypothesis – Chapter 5:

Inhibition of p38 MAPK will inhibit AMPK, attenuate glycolysis and H^+ production, and thus restore the cardioprotective effects of adenosine in stressed hearts during reperfusion following severe ischemia.

1.19 Objectives

To simultaneously characterize cardiac function and metabolism; as well as indices of AMPK and p38 MAPK activity in response to treatment with p38 MAPK inhibitors in the isolated working rat heart model during aerobic perfusion and reperfusion following ischemia.

1.20 Specific Aims

1. To characterize the effects of inhibiting MAPKs (ERK 1/2 or p38 MAPK) on the recovery of left ventricular function following hypothermic ischemia.

2. To characterize the involvement of AMPK in mediating the adenosine-induced stimulation of glycolysis and H^+ production following transient ischemia (two 10-min periods of ischemia and 5-min reperfusion).

3. To characterize the involvement of p38 MAPK in mediating the adenosine-induced stimulation of glycolysis and H^+ production following transient ischemia.

4. To characterize the effects of inhibiting p38 MAPK and AMPK in a model transient, antecedent ischemia on the cardioprotective and metabolic effects of adenosine.

Figure 1-1: Schematic representation of the catabolism of glucose via glycolysis.

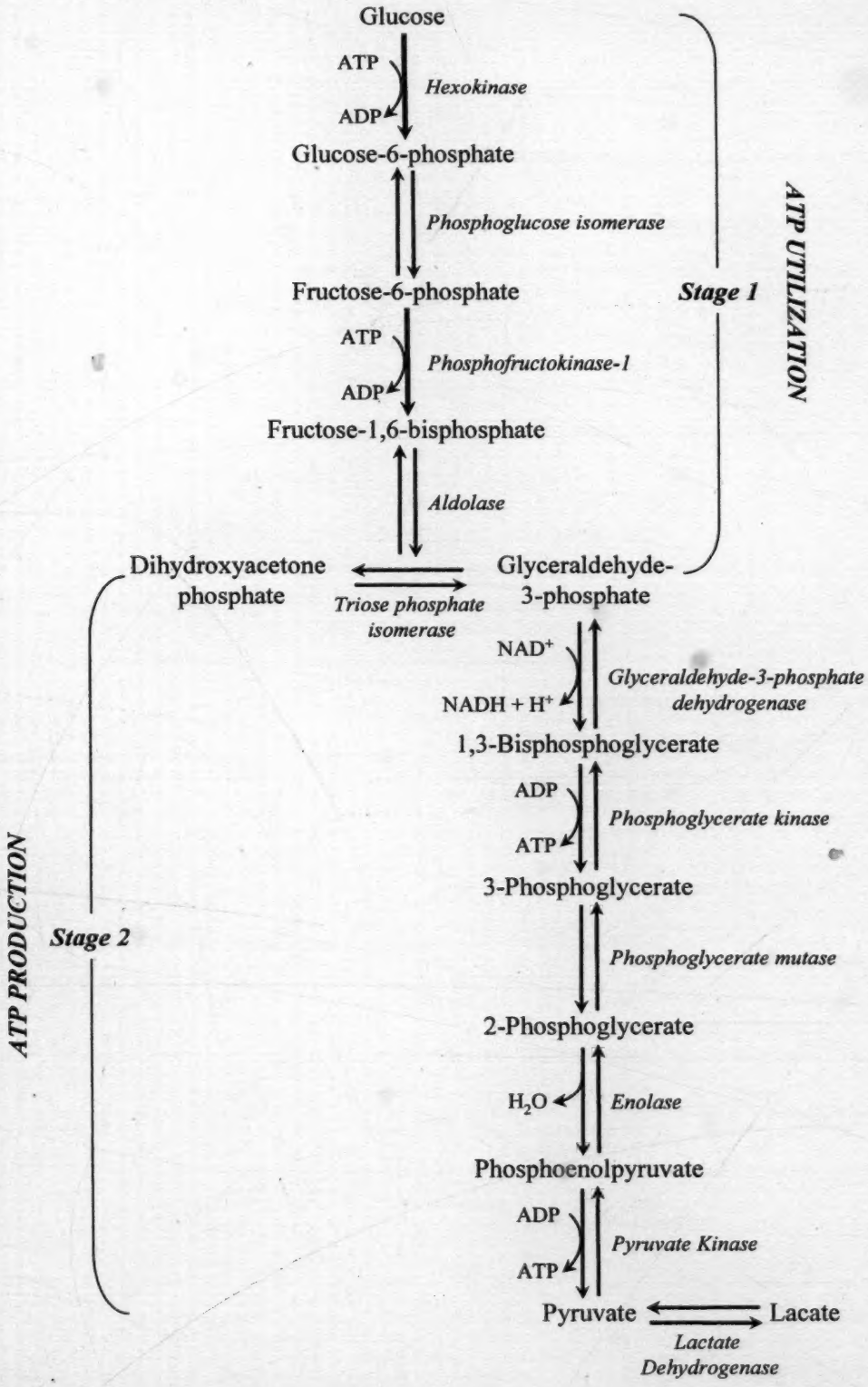


Figure 1-2: Schematic representation of the oxidative decarboxylation of pyruvate (glucose oxidation)

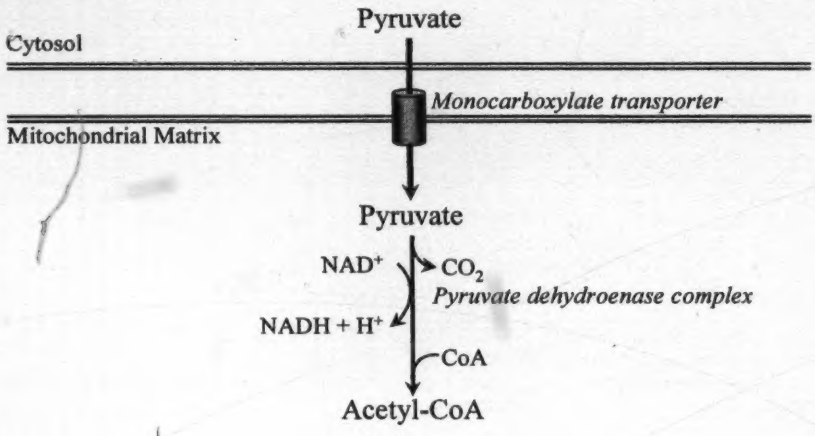


Figure 1-3: Schematic representation of the catabolism of fatty-acyl-CoAs by mitochondrial β-oxidation.

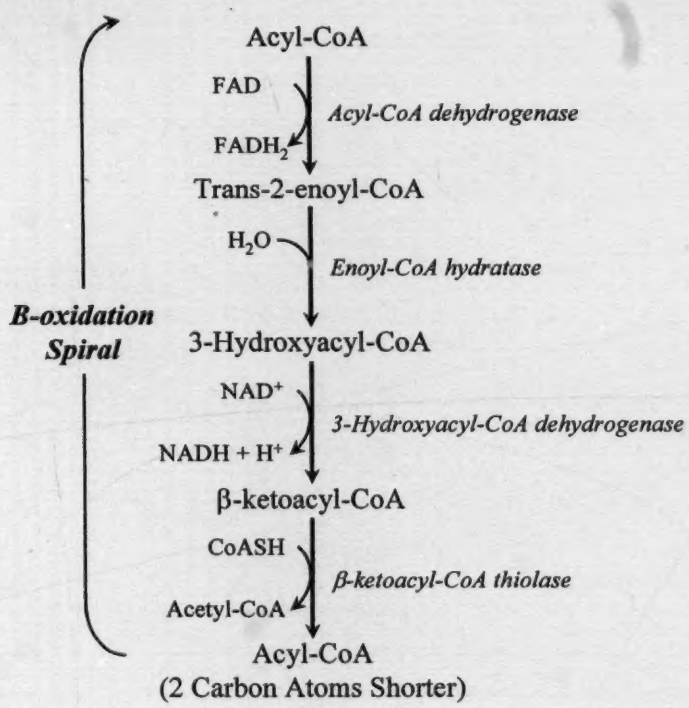


Figure 1-4: Schematic representation of the reactions of the tricarboxylic acid cycle.

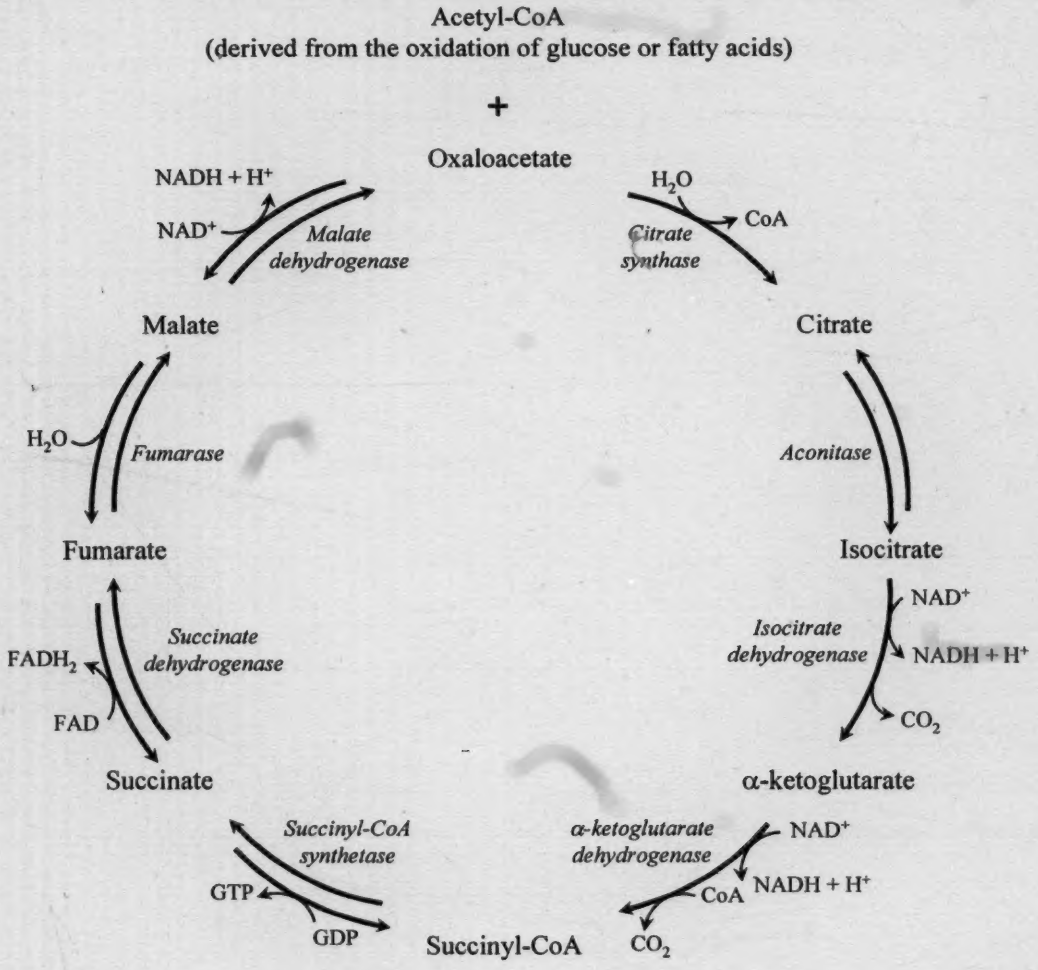
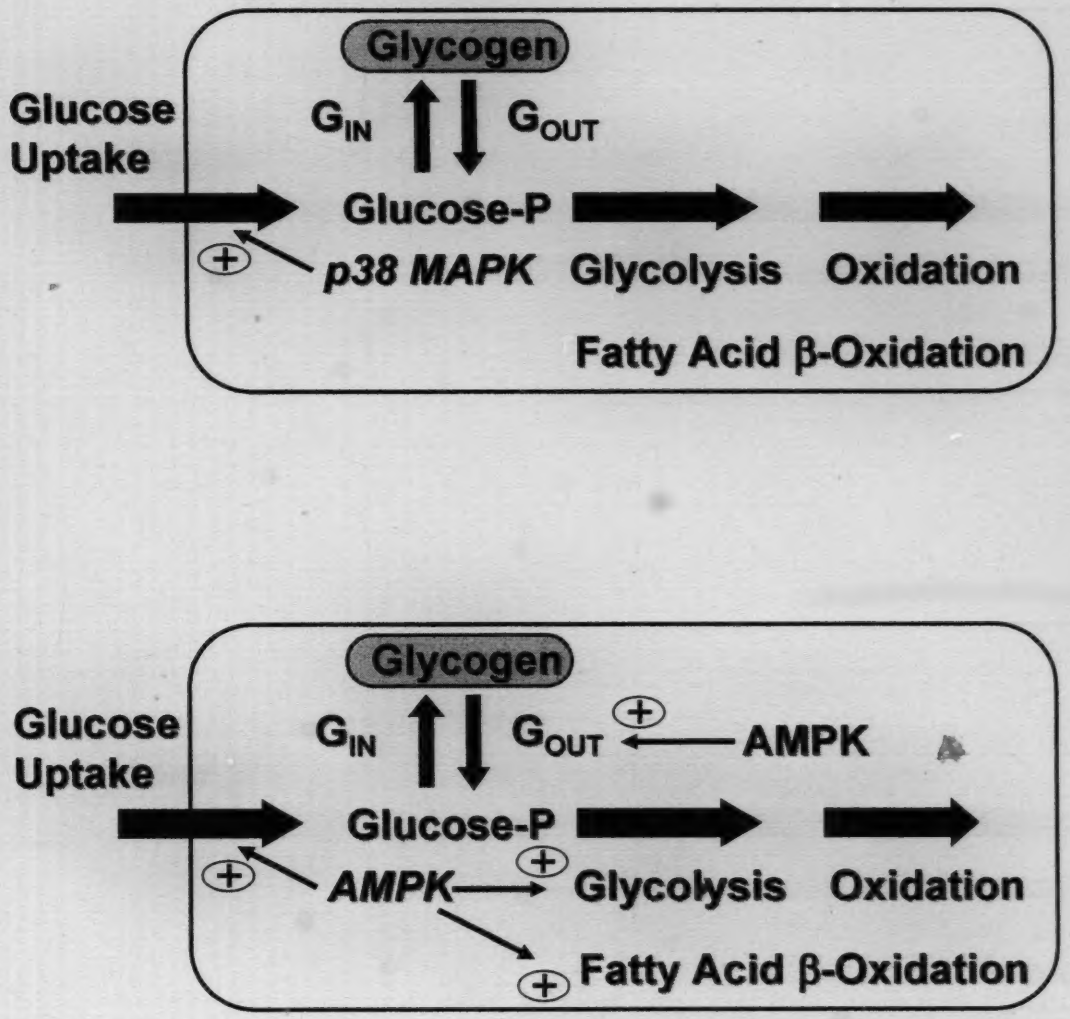


Figure 1-5: Schematic representation of the effects of p38 MAPK and AMPK on glucose metabolism (glycolysis, glucose oxidation, and the net rates of glycogen synthesis (G_{IN}) and glycogen degradation (G_{OUT})), and fatty acid β-oxidation.



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CHAPTER 2

Effects of Inhibition of Myocardial ERK and p38 MAPK on Mechanical Function of Rat Hearts Following Prolonged Hypothermic Ischemia

Cell culture experiments and immunoblot analysis was performed by members of Dr. J.C. Stone's laboratory.

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2.1 Introduction

Mitogen-activated protein kinase (MAPK) cascades serve to link changes in external cell environment to altered intracellular biochemical activities and appropriate cellular responses (Figure 2-1). In the extracellular-responsive kinase (ERK 1/2) system, plasma membrane receptor events are coupled to intracellular tyrosine kinases and the small GTPase, Ras [1]. Ras activation triggers the Raf-Mek-Erk protein kinase cascade. The p38 MAP kinase signaling system also involves a kinase cascade, involving mitogen-activated protein kinase activated protein kinase 2 (MAPKAPK2) as a downstream target [1]. A common feature of regulation of each MAP kinase is dual phosphorylation of their activation loop by upstream kinases (Figure 1). Once activated, MAP kinases are capable of phosphorylating downstream targets including other protein kinases and transcription factors [1,2].

Activation of the ERK system is generally anti-apoptotic [1], is associated with proliferative responses and can cause malignant transformation. It also plays a role in drug-induced [3] and ischemia-induced preconditioning [4]. The p38 MAPK system is activated in response to various stresses, including osmotic stress, and activates apoptosis [5] and depresses myocardial contractility [6]. This distinction between the ERK and p38 MAPK is not absolute, however, and ERK activation has also been associated with reduced cell viability and enhanced apoptosis [7,8]. The basis for this complexity may stem from the existence of multiple and often shared upstream regulatory kinases [9]. It is presently unclear how various types of stress such as hypothermia and ischemia interact to trigger kinase responses.

It has recently reported that hypothermic stress can activate Ras signaling in rat2 fibroblasts [7]. Following exposure of these cells to low temperature, Ras-GTP accumulated, but Raf was not activated. When the cells were re-warmed, the Raf-Mek-ERK cascade was activated, prior to the rapid conversion of Ras-GTP to its inactive GDP-bound form. The response at the level of ERK was documented in a variety of cultured vertebrate cells and we were able to show that ERK activation was detrimental to cell viability; inhibition of activation of the upstream kinase, MEK, with PD98059 increased cell viability following hypothermic stress. We also confirmed the work of others [10,11] that had shown that the early events following acute growth factor stimulation, for example receptor autophosphorylation, proceed efficiently at low temperature.

Hypothermia is a nearly ubiquitous form of environmental stress and so it seems likely that all cells have particularly critical targets for hypothermic stress, as well as sophisticated regulatory mechanisms to deal with reduced metabolic activity. Hypothermia is also an established strategy for tissue preservation *ex vivo*, where the potential damage due to hypothermia *per se* is generally less than the adverse effects of prolonged ischemia. Hypothermic ischemia at < 4°C is essential during donor heart procurement and during storage and transportation prior to transplantation. Current methods for tissue preservation at low temperatures (0 to 4 °C) are largely empirical and provide only short-term protection against the combined effects of prolonged hypothermic and ischemic stress. While hypothermia limits myocardial energy utilization and ischemic injury by depressing the major determinants of myocardial oxygen demand (e.g., tension development, contractility and heart rate), the combination of prolonged hypothermic storage and ischemia induces an unavoidable contractile

dysfunction in human myocardium that limits the optimal storage time to less than 6 hrs [12]. Thus, additional refinements in myocardial protection are necessary in order to extend the safe ischemic time of cardiac allografts.

Considering these points, we were interested to determine whether ERK and p38 MAPK are activated in hearts subjected to cardioplegia and prolonged hypothermic ischemia and whether such changes affect the recovery of mechanical function upon normothermic reperfusion. We report that p38 MAPK is activated by hypothermic stress in perfused rat hearts as well as rat2 fibroblasts and other cell types. We also provide evidence that the detrimental effects associated with this response can be ameliorated by pharmacological inhibition of p38 MAPK with SB202190, a highly selective inhibitor of p38 MAPK activity [13]. Recently, another inhibitor of p38 MAPK was reported to enhance the viability of rat liver transplanted following prolonged hypothermia [14].

2.2 Materials and Methods

2.2.1 Heart Extraction and Perfusion

All animals received care according to the Canadian Council on Animal Care and the University of Alberta Health Sciences Animal Welfare Committee. The procedure for the perfusion of isolated rat hearts has been described by us previously [15,16]. Briefly, hearts were extracted from pentobarbital anesthetized male Sprague-Dawley rats (300 to 350 g) and perfused without pacing in the Langendorff (non-working) mode at a constant aortic perfusion pressure of 60 mm Hg for 10 min with Krebs-Henseleit solution (37°C, pH 7.4, gassed with 95% O_2 / 5% CO_2 mixture) containing 11 mM glucose and 2.5 mM Ca^{2+}.

2.2.2 Hypothermia-Induced Alterations in Heart Function

Following the 10-min period of stabilization, hearts were either perfused in working mode (30 min) to assess normal aerobic function in the absence of hypothermia or ischemia (Fresh group) or were arrested with a clinically useful cardioplegic solution (25 ml of ice-cold St. Thomas's II solution with the following composition: 110 mM NaCl, 10 mM $NaHCO_3$, 16 mM KCl, 16 mM $MgCl{\cdot}6H_2O$, 1.2 mM $CaCl_2$, pH 7.8, ungassed) delivered into the aorta at a constant perfusion pressure of 60 mmHg (Hypothermia group). Arrested hearts were then removed from the perfusion rig and subjected to hypothermia by immersion storage in St. Thomas's II solution for 8 hr at 3°C. Thereafter, to mimic the clinical conditions of re-warming and reperfusion following prolonged hypothermic ischemia, hypothermic hearts were re-warmed without pacing during a 10-min period of Langendorff (non-working) reperfusion with Krebs-Henseleit solution at 37 °C and then subjected to aerobic working mode reperfusion for 30 min (Figure 2-2).

2.2.3 Assessment of Mechanical Function

Mechanical function was assessed in Fresh and Hypothermia groups during perfusion in the working mode at a constant left atrial preload (11.5 mmHg), aortic afterload (80 mmHg), heart rate (pacing at 300 beats/min) and temperature (37 °C). The reperfusion perfusate (recirculating volume of 100 ml) consisted of a modified Krebs-Henseleit solution containing 2.5 mM Ca^{2+}, 11 mM glucose, 100 $mU.L^{-1}$ insulin, and 1.2 mM palmitate pre-bound to 3% bovine serum albumin (BSA, fraction V).

During working mode perfusion, systolic and diastolic aortic pressures were measured with a Gould P21 pressure transducer attached to the aortic outflow line.

Ultrasonic flow probes (Transonic T206) were placed in the left atrial inflow line and the aortic outflow line and were used to measure cardiac output ($ml.min^{-1}$) and aortic flow ($ml.min^{-1}$), respectively. Coronary flow was calculated as the difference between cardiac output and aortic flow. LV work, that served as a continuous index of mechanical function, was calculated as cardiac output X [systolic pressure - left atrial preload]. Hypothermic stored hearts either received drug vehicle (dimethylsulphoxide (DMSO)) at a final concentration of 0.4% for periods equivalent to each of the drug treatments (Untreated) or were treated with either an upstream inhibitor of ERK, PD98059 (20 μM), during storage and re-warming, or the p38 MAPK inhibitor, SB202190 (10 μM) either only during the 10-min period of Langendorff rewarming (SB-LR) or only during working reperfusion (SB-WR) (Figure 2A). PD98059 (Cat# 506126) and SB202190 (Cat# 559388) were purchased from Calbiochem (La Jolla, CA) and were initially dissolved in DMSO before dilution to their final concentrations (20 μM and 10 μM, respectively) in either the storage or perfusate solutions. The final DMSO concentration (0.4%) has no effect on mechanical function of the isolated working heart.

2.2.4 Assessment of ERK and p38 MAPK Activities

Hearts were rapidly frozen with Wollenberger clamps cooled to the temperature of liquid N_2 and stored at -80°C for subsequent biochemical analysis. Measurements were made on hearts (Figure 2B) after the initial Langendorff period of stabilization (Fresh), on hearts after cardioplegic arrest and prolonged hypothermic storage (End Hypothermia) or on stored hearts that had undergone re-warming followed by working mode reperfusion either in the absence (Untreated) or presence of SB202190 (10 μM) (SB-WR). For the immunoblotting procedures, protein lysates (100 μg soluble protein

per sample) of heart extracts were resolved by electrophoresis using 10% sodium-dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). After blotting onto a nitrocellulose membrane, the activated forms of protein kinases were detected with phospho-specific antibodies (Cell Signaling Technologies, Beverly, Mass.) that recognized the phosphorylated form of p38 MAPK (# 9211). Antibodies that recognize both phosphorylated and non-phosphorylated forms of p38 MAPK (#9212) were used to confirm equivalent amounts and integrity of protein among samples within each experiment. Membranes were also stained with Ponceau S to verify equivalent protein loading. Signals were developed by enhanced chemiluminescence (Supersignal Picokit, Pierce Technologies). The activity of the kinase MAPKAPK2, a downstream target of p38 MAPK, was measured using an immune complex kinase assay (Upstate Biotechnology, Lake Placid, NY) according to the supplier's instructions.

2.2.5 Cell Culture

Most of the cells and their methods of culture have been described previously [7]. PC12 cells were maintained in medium containing 10% horse serum plus 5% calf serum. For each comparison, 25 cm^2 flasks were seeded with equal numbers of cells and grown for 2-3 days in a 10% CO_2 environment at which point they were near, or at, saturation density.

2.2.6 Hypothermia-Induced Alterations in Cultured Cells

To examine in more detail the influence of hypothermia on p38 MAPK signaling, we exposed rat2 cells (embryonic rat fibroblast cell line) to 0°C for graded periods of time and following re-warming to 37°C. Proteins in total cell lysates were resolved by SDS-PAGE and probed with antibodies that recognize the activated, phosphorylated forms of

various stress kinases. Control flasks were either untreated or were exposed to medium containing 600 mM sorbitol at 37°C for one hour to elicit osmotic stress that served as a standard stimulus for p38 MAPK activation. Experimental flasks were tightly capped to maintain the CO_2 environment and then placed on ice to induce hypothermic stress. After graded periods, culture medium was aspirated on ice and cells were lysed in a SDS-containing sample buffer. Immunoblotting procedures on cultured cells were performed as described above for heart extracts.

2.2.7 Statistical Analysis

Data are expressed a mean ± SE. Differences among groups were analyzed by One-way analysis of variance (ANOVA) and if significant followed by Bonferroni post-hoc tests. A P value of < 0.05 was considered statistically significant.

2.3 Results

2.3.1 Effect of Prolonged Hypothermia on Recovery of Mechanical Function

In hearts subjected to cardioplegic arrest, hypothermic storage at 3°C for 8 hrs, re-warming for 10 min in Langendorff mode and then subjected to 30 min working mode reperfusion, LV work recovered to 23% (n=12) of the LV work level measured in Fresh hearts (n=6) (Figure 2-3). The other indices of mechanical function are shown in Table 1 and indicate that prolonged hypothermia had marked deleterious consequences on post-storage mechanical function.

2.3.2 Effect of ERK Inhibitor on Recovery of Mechanical Function

PD98059, when present during hypothermic storage, Langendorff re-warming and working mode reperfusion, did not significantly affect the recovery of LV work or any of the other indices of mechanical function (data not shown). However, when

PD98059 was present only during the storage and re-warming phases (Figure 2-3A), there was a modest enhancement in the recovery of LV work to 43% the values observed in Fresh hearts. Recoveries of cardiac output and aortic flow were also significantly enhanced when PD98059 was present during the storage and re-warming periods (Table 1).

2.3.3 Effect of p38 MAPK Inhibitor on Mechanical Function

SB202190 (10 μM), when present only during the 10-min period of Langendorff re-warming, had no effect on the extent of recovery of LV work (18%) (Figure 2-3B) or other indices of mechanical function (Table 2-1). However, when SB202190 (10 μM) was present only during the period of working reperfusion, there was an immediate and sustained increase in the recovery of LV work to 73% of values observed in Fresh hearts (Figure 3B). Improvements in the recovery of the other indices of myocardial function were also noted (Table 2-1). Coronary vascular resistance was not affected, indicating that SB202190 did not elicit its beneficial effects via increases in coronary perfusion. SB202190 did not affect mechanical function of aerobic hearts, nor did it alter the rates of glucose metabolism (Table 2-2).

2.3.4 Phosphorylation of Myocardial p38 MAPK by Hypothermic Stress

To confirm that the mechanism of the beneficial effects of SB202190 was related to p38 MAPK inhibition, the presence of phospho-p38 MAPK, indicative of the activated form of p38 MAPK, was assayed in hearts harvested at time-points within the perfusion protocols (Figure 2-2). Protein extracts from Fresh hearts prior to working mode perfusion contained a basal level of phospho-p38 MAPK (Figure 2-4A). The level of phosphorylation increased after 8 hrs of hypothermic ischemia, but did not return to basal

levels after re-warming. Indeed, the highest content of phospho-p38 MAPK was observed in Untreated hearts re-warmed for 10 min and then reperfused for 30 min in working mode (Figure 2-4B). Since we were interested in determining the possible consequences of pharmacological inhibition of p38 MAPK activity, we also measured phospho-p38 MAPK in hearts that had been reperfused in the presence of SB202190. This drug is thought not to interfere with p38 MAPK phosphorylation, but rather inhibits the catalytic activity of the phosphorylated enzyme. Unexpectedly, however, the level of phospho-p38 MAPK was lower when the inhibitor was present.

2.3.5 Activation of Myocardial MAPKAPK2 in Response to Hypothermic Stress

To explore the biochemical relevance of hypothermia-induced phosphorylation of p38 MAPK, we measured the activity of MAPKAPK2, a downstream protein kinase phosphorylated and activated by p38 MAPK that can catalyze the phosphorylation of additional downstream targets such as heat shock protein-27 (HSP-27) [17]. In extracts of hearts from the Fresh group, basal MAPKAPK2 activity was readily detectable (Figure 2-4C). After prolonged hypothermia, but before re-warming, MAPKAPK2 activity was not increased despite the marked activation of p38 MAPK. However, following re-warming and normothermic reperfusion in working mode, heart extracts displayed a marked increase in MAPKAPK2 activity. Importantly, this activation of MAPKAPK2 was prevented by the p38 MAPK inhibitor, SB202190 (Figure 2-4C).

2.3.6 Phosphorylation of p38 MAPK in Cultured Cells in Response to Hypothermic Stress

To explore the generality of our observations on p38 MAPK activation, we sought to test the response in other cell types. The phosphorylated form of p38 MAPK

was not detectable in confluent, normothermic rat2 fibroblasts (Figure 2-5). As expected, phospho-p38 MAP kinase was readily detectable after exposure of rat2 cells to hyperosmotic stress with sorbitol (600 mM for 1 hr at 37°C). Hypothermic stress induced a reproducible and biphasic phosphorylation of p38 MAPK. After an initial modest accumulation of phospho-p38 MAPK within the first 30 min, the level decreased to basal values after 2 and 4 hrs of hypothermia. However, after prolonged hypothermia (8 hrs), a robust level of phospho-p38 MAPK was detectable, that was further elevated during the first 15 min of re-warming before returning to basal levels. By probing these lysates with an antibody that recognizes both the phosphorylated and non-phosphorylated forms of p38 MAPK, we were able to show that these changes largely reflected phosphorylation and de-phosphorylation reactions, as the amount of total cellular p38 MAPK was comparable among samples (Figure 2-5A).

The phosphorylation of p38 MAPK in response to hypothermic stress appeared to be relatively specific, as no phosphorylation of p54 or p46 species of JNK/SAPK was observed in these lysates (Figure 2-5B). Likewise, MKK4, a normal activator of JNK/SAPK also did not exhibit increased phosphorylation in cells subjected to hypothermic stress (Figure 2-5B). Additional studies in rat2 cells indicated the presence of detectable MAPKAPK2 activity under normothermic conditions. Despite marked phosphorylation of p38 MAPK during prolonged hypothermia (15 hrs), only modest activation of MAPKAPK2 was demonstrable. However, following re-warming, as in Untreated perfused hearts, the consequences of p38 MAPK activation become manifest as a marked activation of MAPKAPK2 (Figure 2-5C).

We also studied hypothermia-induced activation of p38 MAPK in other cell types (Figure 2-6). These included HEK 293 (adenovirus transformed human kidney), PC12 (rat pheochromocytoma), KD (normal human fibroblast), NIH3T3 (mouse embryonic fibroblast), COS7 (African Green Monkey kidney), and CEF (chicken embryo fibroblast). Responses of cells that were exposed to both brief (30 min) and prolonged (8 hrs) periods of hypothermia were compared with cells that were either untreated (normothermic) or exposed to osmotic stress (600 mM sorbitol). While brief hypothermia (30 min) increased phospho-p38 MAPK levels in most cell types, prolonged hypothermia (8 hrs) increased levels of phospho-p38 MAPK, in most cases, to levels equivalent to that seen in response to osmotic stress. To gain further insight into the mechanism underlying p38 MAPK phosphorylation, we probed the same lysates with an antibody that recognizes the phosphorylated forms of MKK3 and MKK6, both upstream activators of p38 MAPK. In some cells (e.g. COS and CEF), the pattern of MKK3/6 phosphorylation mirrored that of p38 MAPK. In others, hypothermic activation of MKK3/6 was not obvious (Figure 2-6).

2.4 Discussion

2.4.1 Summary of Major Findings

Heart transplantation is a life saving, therapeutic modality for patients with end stage heart disease but, unfortunately, the optimal storage time for donor hearts remains less than 6 hours, thereby limiting long distance organ procurement [12]. While myocardial preservation techniques have evolved in conjunction with cardiac surgery and at present offer significant protection against cardiac injury, remarkably, very little is known about the specific biochemical changes that attend prolonged hypothermic storage of hearts and other organs and tissues used in clinical transplantation. Clearly, a better understanding of

changes and associated deleterious effects will lead to the development of therapeutic interventions to facilitate organ retrieval and to enhance contractile recovery following prolonged hypothermia. This study investigated whether hypothermia-induced activation of ERK or p38 MAPK signaling occurred in rat hearts under conditions comparable to those used in clinical transplantation. We demonstrate a marked activation of p38 MAPK signaling in hearts that were subjected to cardioplegia, prolonged hypothermic storage, re-warming and reperfusion. Importantly, inhibition of p38 MAPK during reperfusion resulted in an immediate and sustained improvement in mechanical function, a beneficial effect that suggests that a more complete understanding of hypothermia-induced activation of MAP kinase signaling could lead to improved methods for cryopreservation of clinical material.

2.4.2 Hypothermic-Ischemia and ERK Activation

Several studies have addressed MAP kinase signaling in hearts subjected to normothermic ischemia or oxidative stress [18,19,20] where activation of different members of the MAPK superfamily, including ERK, p38 MAPK and JNK/SAPK have been documented. The activation of the ERK signaling cascade by oxidative stress with H_2O_2 limits cellular injury by induction of cytoprotective cyclooxygenase-2 (COX-2) [21]. Moreover, selective inhibition of ERK activation with PD98059 worsens oxidative damage [22] and reperfusion injury [20]. The ERK pathway is also implicated in the regulation of inducible nitric oxide synthase (iNOS) [23] and may exert protective actions by correction of deficiencies in nitric oxide (NO) biosignaling following prolonged hypothermia [16], in a manner similar to the beneficial actions of exogenous NO donors [15]. In contrast, adverse effects of ERK activation have been noted following hypothermia

where ERK inhibition with PD98059 enhanced cell viability [7]. In this study, the inhibitor of ERK activation, PD98059, showed only modest cardioprotective efficacy in hearts subjected to prolonged hypothermia, probably reflecting the combination of beneficial and adverse consequences of ERK activation.

2.4.3 Hypothermic Ischemia and p38 MAPK Activation

The roles of p38 MAPK in normothermic ischemia are also controversial and both deleterious (increased myocyte apoptosis) and beneficial effects (preconditioning) have been reported [6,18,19,20]. While its role in hypothermic hearts has hitherto not been studied, a recent report indicates that it is activated, and elicits deleterious effects, during re-warming following prolonged hypothermia in liver [14]. Moreover, p38 MAPK activation is associated with depressed cardiomyocyte shortening, suggesting a role in the control of cardiac mechanical function [6]. We now provide evidence that prolonged hypothermic stress also activates p38 MAPK in perfused hearts as well as in a variety of cells in culture. Moreover, we demonstrate that hypothermia-induced activation of p38 MAPK in the myocardium has adverse consequences as inhibition of p38 MAPK enhances the recovery of mechanical function following re-warming. In our study, the effectiveness of the p38 MAPK inhibitor was confirmed by assay of its downstream target, MAPKAPK2. The inability of the p38 MAPK inhibitor to enhance recovery of mechanical function when present only during the short 10-min period of re-warming indicates that the alterations in downstream signaling events responsible for enhanced recovery are operative during working mode reperfusion. The precise mechanisms of protection were not identified in the present study, but it is clear that improved contractile function was not due to a direct positive inotropic action of SB202190 as this agent does not alter mechanical function of

aerobically perfused hearts. Signaling via p38 MAPK is involved in apoptosis, but the immediate functional benefit arising from inhibition of p38 MAP kinase and MAPKAPK2 argues in favor of a post-translational (non-genomic) mechanism involving either enhanced energy production or improved energy utilization. Moreover, the ability of post-ischemic drug administration that inhibits p38 MAPK or that enhances NO biosignaling [15] to elicit rapid improvement of post-ischemic mechanical function suggests that a significant component of the mechanical dysfunction observed following prolonged hypothermic ischemia is reversible, and therefore amenable to therapeutic intervention.

Other investigators have noted changes in stress kinase activity during and after recovery from hypothermic stress. For example, p38 MAPK phosphorylation was noted in bronchial epithelial cells subjected to hypothermia (0°C) and this response was linked to stimulation of IL-8 expression after re-warming to 37°C [24]. However, the generality of the p38 MAPK response during hypothermia was not previously appreciated. Our recent serendipitous discovery [7] that ERK was activated in many cultured cell types immediately after re-warming from hypothermia lead to our discovery that p38 MAPK is robustly phosphorylated in response to prolonged hypothermia in all cell lines tested, while JNK/SAPK is not.

2.4.4 Mechanism of p38 MAPK Activation in Response to Hypothermic Ischemia

The mechanism underlying p38 MAPK activation is not clear. In principle, p38 MAPK phosphorylation could arise from the hypothermic activation of upstream activating kinases such as MKK3 or MKK6, or from low basal phosphorylation in the face of hypothermic inhibition of p38 MAPK phosphatase, or both. Previously, we and

others have provided evidence that growth factor-receptor interactions at 0°C can lead to the activation of tyrosine kinases and other signaling components that positively regulate Ras, providing a precedent for the efficient functioning of upstream signaling molecules in the ERK system at low temperature [1,10,11]. This study now extends those observations and indicates that p38 MAPK is also activated under conditions of hypothermic stress. Moreover, our results with the phospho-MKK3/6 antibody show that prolonged hypothermia results in the phosphorylation of at least one of these upstream p38 MAPK activators in various cell types. Exposure to hyperosmolar conditions is expected to lead to cell shrinkage while hypothermia leads to cell swelling, suggesting that p38 MAPK activation in each case may arise from osmo-mechanical changes at the plasma membrane. However, osmotic stress with sorbitol activated both p38 MAPK and JNK/SAPK, while hypothermic stress activated only the former. Thus, hypothermia does not simply mimic osmotic stress as it involves mechanisms specific to the p38 MAPK pathway. Interestingly, activation of both ERK and p38 MAPK have been observed in a variety of cells including immortalized cell lines derived from various mammalian, avian and insect sources. In contrast, not all cell lines exhibited ERK activation [7]. Notably, we were unable to demonstrate ERK activation in NIH3T3 cells or PC12 cells during re-warming, although the present study indicates that both cell types responded well at the level of p38 MAPK phosphorylation. The potential mechanistic relationship between p38 MAPK and ERK signaling in response to hypothermic stress remains to be explored.

2.4.5 Conclusions

Pharmacological inhibition of either ERK [7] or p38 MAPK ameliorates injury following hypothermia and rewarming. At face value, these results indicate that

hypothermia-induced activation of MAPK signaling is detrimental to cells, raising questions about its adaptive significance. The existence of such systems might be rationalized in terms of a threshold model. MAPK responses may be beneficial to the cell after mild hypothermia. After more prolonged or more severe hypothermic stress, MAPK signaling may contribute to orderly cell death, perhaps orchestrated by the apoptotic machinery. The advantages of such systems might out-weigh the inherent risk of organismal death after lethal exposure to low temperature. Thus, one of the primary adaptive functions of the p38 MAPK system, and perhaps the Ras-ERK system, may be to allow the organism to deal with the nearly ubiquitous environmental threat of hypothermia.

Whatever the normal significance of ERK and p38 MAPK signaling in response to hypothermic stress, we have argued that a fuller understanding of these processes could have important implications for the hypothermic storage of clinical material used in transplantation scenarios. While hypothermia is often used to reduce metabolic demand and reduce the adverse effects of ischemia, cooling is effective for only limited periods of time. In heart transplantation, graft survival is best achieved if the organ is subjected to less than 6 hrs of hypothermic storage. Beyond this time, myocardial injury seriously limits the recovery of contractile function of the human heart [25]. Hopefully, a better understanding of the biochemical processes underlying these deleterious effects will lead to the development of therapeutic interventions to facilitate organ retrieval and to enhance recovery following hypothermia. Our demonstration that inhibition of p38 MAPK contributes to an improved recovery of cardiac mechanical function following

hypothermic stress suggests that studies on the protective efficacy of inhibitors of p38 MAP kinase signaling in transplant models are warranted.

Figure 2-1. Diagram of the MAP kinase superfamily indicating the ERK, p38 and SAPK/JNK pathways. Osmotic stress activates both the p38 and SAPK/JNK pathways, while hypothermia has been shown to activate the ERK pathway. The effect of hypothermic stress on the p38 pathway, and its potential consequences on cardiac mechanical function following rewarming have not been defined.

Figure 2-1

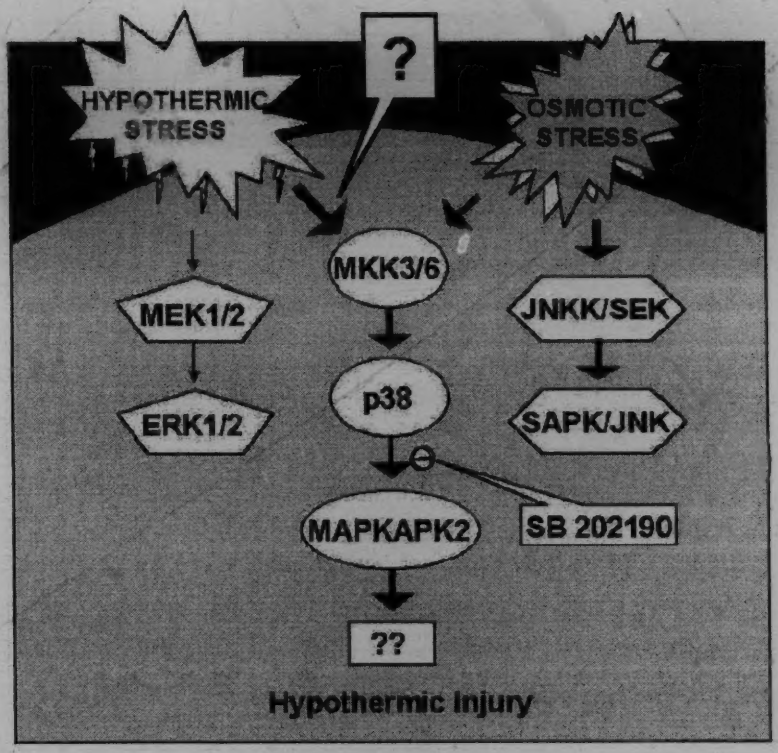
?
HYPOTHERMIC STRESS
OSMOTIC STRESS
MKK3/6
MEK1/2
JNKK/SEK
p38
ERK1/2
SAPK/JNK
MAPKAPK2
SB 202190
??
Hypothermic Injury

Figure 2-2. Diagram of the isolated heart perfusion protocols for functional and biochemical analyses of hearts subjected to hypothermia. A) For Functional Analysis, hearts were perfused in aerobic working mode for 30 min for the measurement of baseline LV work (Fresh Work) or subjected to cardioplegic arrest and 8 hrs hypothermic ischemia at 3 ± 1 °C. Thereafter, these hearts were re-warmed to 37 °C during a 10-min Langendorff mode reperfusion (LR) and were subjected to aerobic working reperfusion (WR). Hearts were either untreated (Untreated-WR) or were exposed to PD98059 (20 μM) during storage and re-warming periods (PD-S-LR), SB202190 (10 μM) either only during the Langendorff re-warming phase (SB-LR) or only during the period of working reperfusion (SB-WR). B) For Biochemical Analysis, hearts were rapidly frozen either after an initial 10-min Langendorff stabilization period (Fresh) or after being subjected to cardioplegic arrest and 8 hrs hypothermic ischemia at 3 ± 1 °C (End Hypothermia). Additional groups were frozen after rewarming and working reperfusion (WR) either in the absence (Untreated-WR) or in the presence of SB202190 (10 μM) (SB-WR).

Figure 2-2

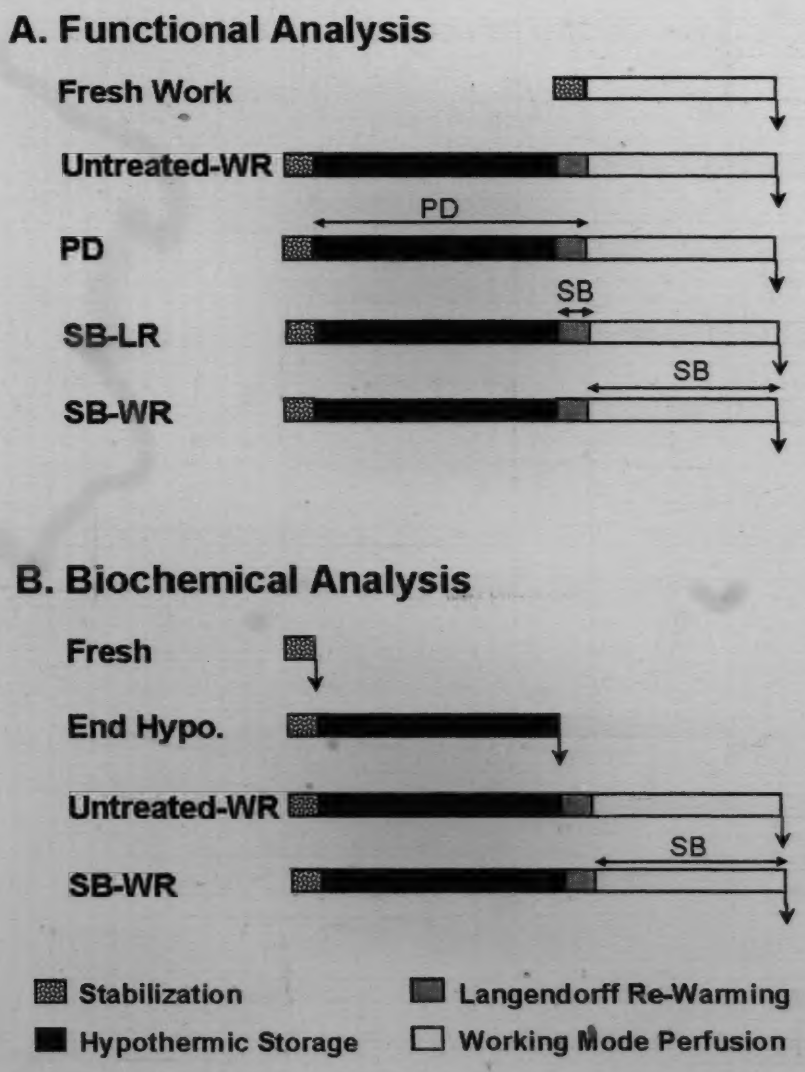
A. Functional Analysis
Fresh Work
Untreated-WR
PD
PD
SB-LR
SB
SB-WR
SB
B. Biochemical Analysis
Fresh
End Hypo.
Untreated-WR
SB-WR
SB
Stabilization
Hypothermic Storage
Langendorff Re-Warming
Working Mode Perfusion

Figure 2-3. Time course of the recovery of mechanical function (LV work) during working mode reperfusion. Data are shown for freshly excised hearts (Fresh, ● , n=4), and for hearts subject to cardioplegic arrest and 8 hrs hypothermic ischemia, Langendorff re-warming and then working mode reperfusion in the absence (Untreated-WR, ■ , n=12) or presence of SB202190 (10 μM) during only Langendorff reperfusion (SB-LR, □ , n=4) or during only working reperfusion (SB-WR, ○, n=6). Data are presented as the mean ± SEM. * indicates $P < 0.05$ relative to Untreated group by ANOVA followed by Bonferroni's 2-tailed t-test.

Figure 2-3

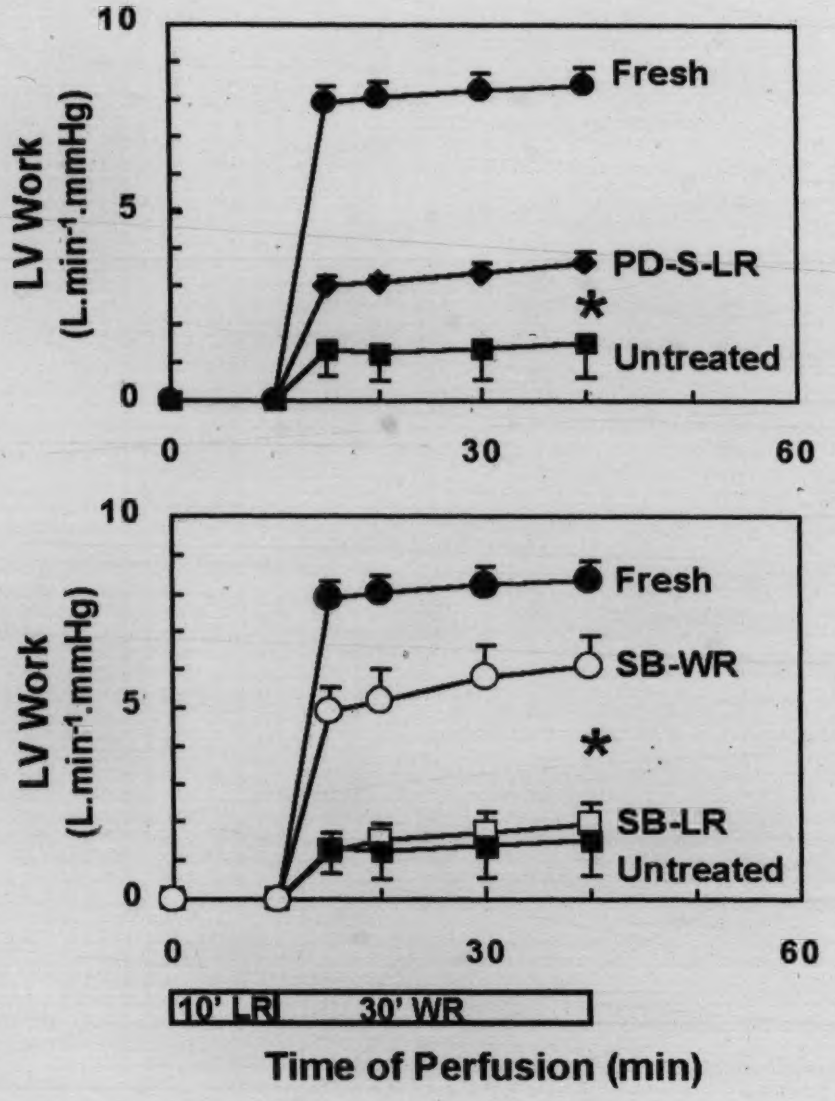
10
5
0
LV Work
(L.min-1.mmHg)
Fresh
PD-S-LR
*
Untreated
0
30
60
10
5
0
LV Work
(L.min-1.mmHg)
Fresh
SB-WR
*
SB-LR
Untreated
0
30
60
10' LR
30' WR
Time of Perfusion (min)

Figure 2-4. Phosphorylation of p38 MAPK and activation of MAPKAPK2 in rat hearts. A) Proteins extracted from frozen powdered heart (100 μg soluble protein per sample) were assayed for phospho-p38 MAPK and total p38 MAPK by immunoblotting. Sample classification is as described in Figure 1B and represents samples of Fresh hearts (FRESH), hearts frozen following hypothermic ischemia for 8 hrs (END HYPO), and for hearts re-warmed and reperfused in working mode (WR) either in the absence (- SB) or presence (+ SB) of SB202190 (10 μM). B) Quantification of phospho-p38 MAPK immunoblots for the same groups of hearts where the band intensities of duplicate heart samples run in adjacent lanes were quantified by densitiometric scanning. C) MAPKAPK2 activities in the same groups of hearts. MAPKAPK2 was immune-precipitated from heart lysates and assayed for catalytic activity using a peptide substrate. Values (c.p.m. ^{32}P incorporated) are mean ± SE for 4 replicate hearts from each group. All values have been corrected by subtracting the average value obtained when primary antibody was omitted.

Figure 2-4

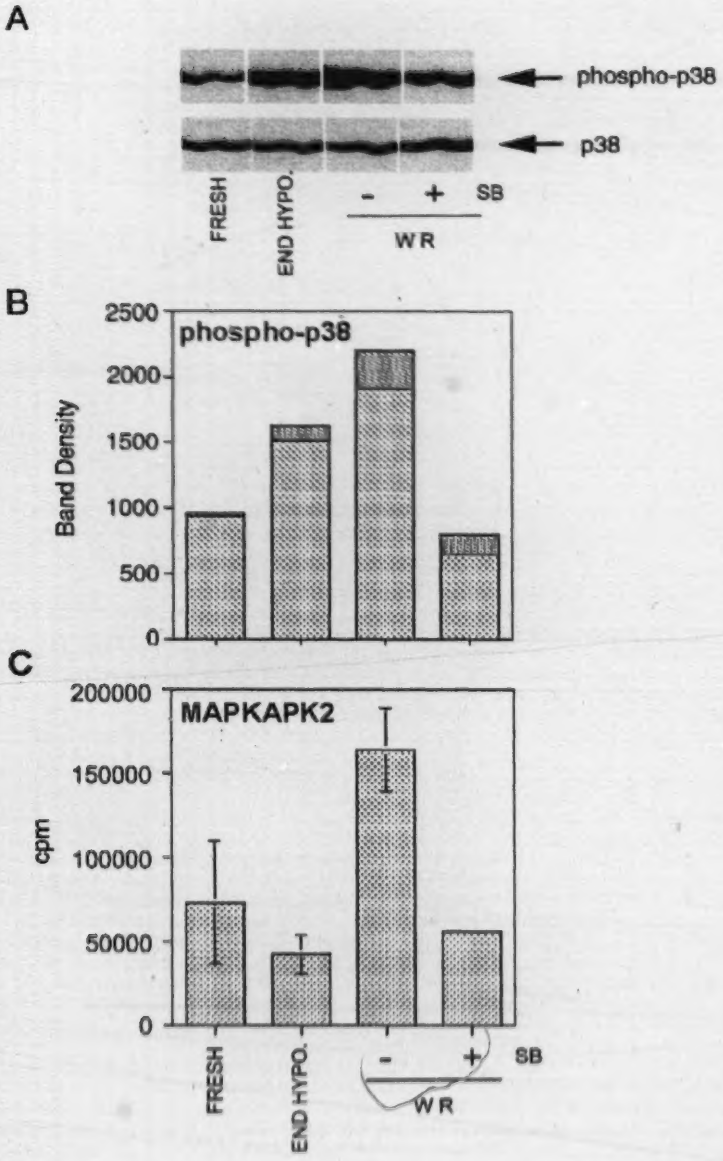
A
phospho-p38
p38
FRESH
END HYPO.
-
+
SB
WR
B
phospho-p38
Band Density
2500
2000
1500
1000
500
0
C
MAPKAPK2
cpm
200000
150000
100000
50000
0
FRESH
END HYPO.
-
+
SB
WR

Figure 2-5. Activation of p38 MAPK in rat2 fibroblasts. A) Cells were either treated with sorbitol (S), left untreated (U) or incubated at 0°C for the indicated times (20 min, 30 min, 1 hr, 2 hrs, 4 hrs or 8 hrs) or for 8 hrs hypothermia followed by warming to 37°C for the indicated times (1 min, 5 min, 15 min, 30 min, 1 hr, 2hrs or 4 hrs). Protein lysates were then resolved by SDS-PAGE and immunoblotted with an anti-phospho-p38 MAPK antibody (Upper Panel). To control for slight variations in cellular protein content among samples, the same lysates we analyzed with an antibody that recognizes total p38 MAPK irrespective of phosphorylation status (Lower Panel). B) Some of the same lysates from above treatment groups were probed with antibodies that recognize the phosphorylated forms of p54 and p46 JNK/SAPK (Upper Panel) or MKK4 (Lower Panel). C) MAPKAPK2 activity, assayed as described for rat hearts, for rat2 cells that were either untreated (U), exposed to hypothermia at 0°C for 15 hrs (HYPO) or exposed to hypothermia for 15 hrs prior to re-warming to 37°C for 15 min (REWARM). As a positive control, cells were also exposed to osmotic stress with sorbitol (S, 600mM for 60 min at 37 °C). Values (c.p.m. ^{32}P incorporated) are mean ± SEM for triplicate determinations. All values have been corrected by subtracting the average value obtained when primary antibody was omitted.

Figure 2-5

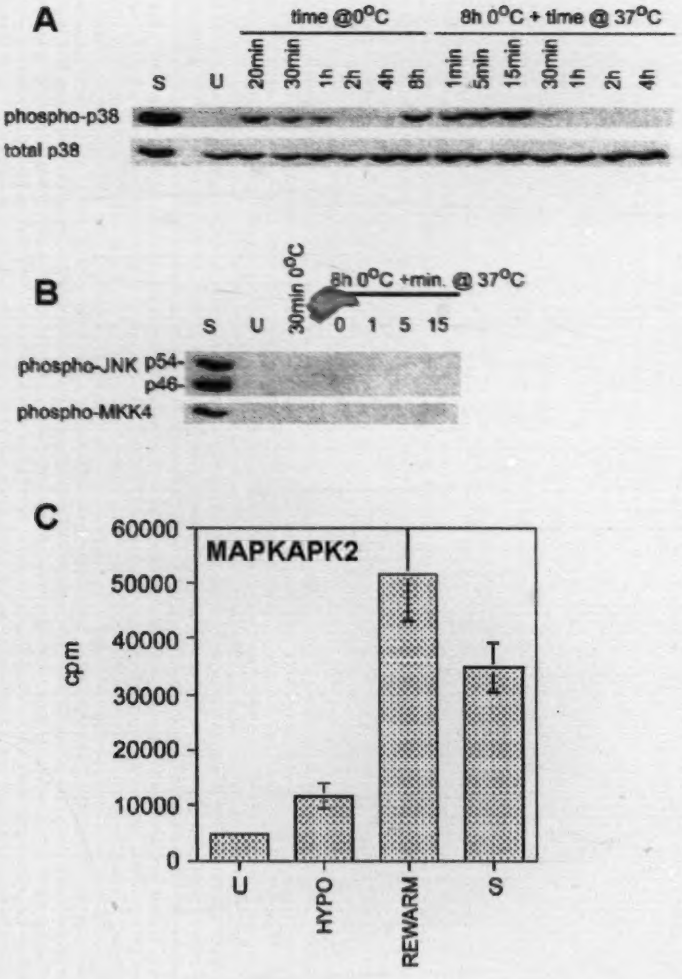
A
time @0°C
8h 0°C + time @ 37°C
S
U
20min
30min
1h
2h
4h
8h
1min
5min
15min
30min
1h
2h
4h
phospho-p38
total p38
B
30min 0°C
8h 0°C +min. @ 37°C
S
U
0
1
5
15
phospho-JNK p54-
p46-
phospho-MKK4
C
MAPKAPK2
cpm
60000
50000
40000
30000
20000
10000
0
U
HYPO
REWARM
S

Figure 2-6. The activation of p38 MAPK (Upper Panel) was examined in various vertebrate cells, including 293 (adenovirus transformed human kidney), PC12 (rat pheochromocytoma), KD (normal human fibroblast), NIH3T3 (3T3, mouse embryonic fibroblast), COS7 (COS, African green monkey kidney), CEF (chicken embryo fibroblast) as well as rat2 cells. Cells were exposed to osmotic stress with sorbitol (S, 600mM for 60 min at 37 °C), left untreated at 37°C (U), or exposed to either brief (30 min) or prolonged hypothermic stress (8 hrs). Cell lysates from these groups were also probed with antibodies that detected the activated versions of MKK3 and MKK6 (Middle Panel) as well as total p38 MAPK irrespective of phosphorylation status (Lower Panel).

Figure 2-6

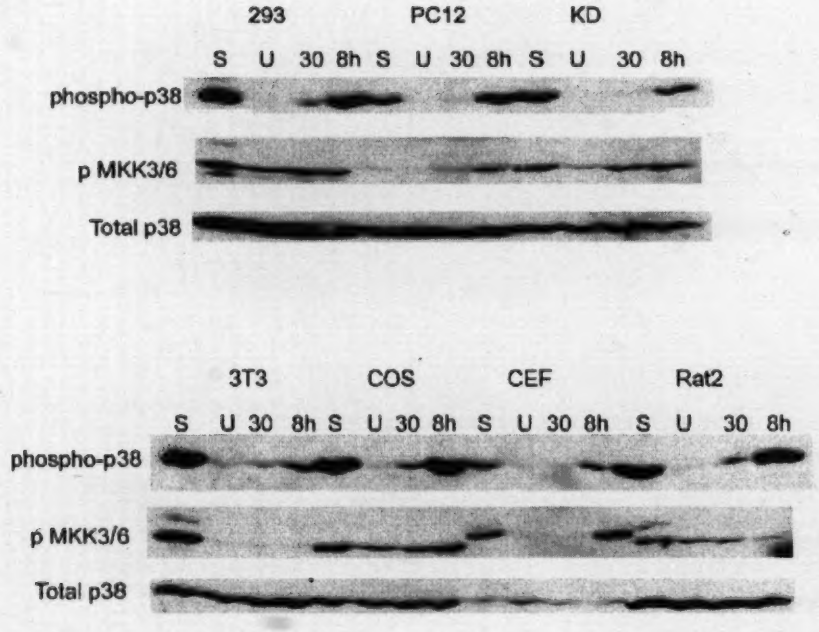
293
PC12
KD
S U 30 8h S U 30 8h S U 30 8h
phospho-p38
p MKK3/6
Total p38
3T3
COS
CEF
Rat2
S U 30 8h S U 30 8h S U 30 8h S U 30 8h
phospho-p38
p MKK3/6
Total p38

Table 2-1. Effect of PD98059 (20 μM) and SB202190 (10μM) on recovery of mechanical function after cardioplegic arrest and prolonged hypothermic ischemia.

Group	n	CO	AoF	CF	CVC	LV Work
Fresh Hearts	6	70.5±2.2	45.3±3.3	25.2±2.5	0.27±0.03	8.37±0.46
Hypothermic Hearts						
Untreated	12	21.8±5.4	7.9±2.3	13.9±2.8	0.18±0.03	1.96±0.53
PD-S-LR	9	37.7±2.4*	18.8±2.3*	18.9±1.3	0.21±0.01	3.66±0.75*
SB-LR	4	17.5±9.0	5.3±3.0	12.3±6.0	0.17±0.07	1.52±0.88
SB-WR	6	57.7±5.3*	36.2±3.6*	21.5±2.3	0.24±0.02	6.12±0.75*

Measurements of cardiac output (CO, $ml.min^{-1}$), aortic flow (AoF, $ml.min^{-1}$), coronary flow (CF, $ml.min^{-1}$), coronary vascular conductance (CVC, $ml.min^{-1}.mm\ Hg^{-1}$) and LV work ($L.min^{-1}.mm$ Hg) were made at the end of working mode perfusion (30 min). Data are presented as the mean ± SE for n observations for Fresh hearts as well as hearts reperfused following prolonged hypothermia. Hypothermic hearts were perfused in the absence (Untreated) or presence of PD98059 (20 μM) during storage re-warming periods (PD-S-LR), SB202190 (10 μM) that was present either only during the 10-min period of Langendorff reperfusion (SB-LR) or only throughout working reperfusion (SB-WR). * indicates $P < 0.05$ by comparison with Untreated-WR hearts (ANOVA).

Table 2-2. Effects of SB202190 (10 μM) on LV work (Joules) and the rates (mmol/g dry wt/min) of glucose metabolism in aerobically perfused rat hearts. Values represent mean±SE, n=5.

Group	LV Work	Glycolysis	Glucose Oxidation	H^+ Production
Vehicle	0.81±0.08	3.59±0.65	1.34±0.29	4.48±0.84
SB202190	0.97±0.06	2.78±0.50	1.03±0.22	3.44±0.78

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Chapter 3

Effects of Adenosine on Myocardial Glucose and Palmitate Metabolism after Transient Ischemia: Role of 5'-AMP-Activated Protein Kinase

HPLC analysis of adenine nucleotides and high energy phosphates was performed by Ken Strynadka.

A portion of the data presented in this chapter has been published as an abstract in *Circulation.* Jaswal JS, Gandhi M, Finegan BA, Dyck JRB, and Clanachan, AS. 108: SIV-18, 2003.

A version of this chapter has been published in the *American Journal of Physiology (Heart Circ Physiol).* Jaswal JS, Gandhi M, Finegan BA, Dyck JRB, and Clanachan AS. 291: H1883-H1892, 2006.

3.1 Introduction

Cardioprotection by adenosine is demonstrable in experimental models of ischemia-reperfusion injury, where it reduces infarct size and improves post-ischemic LV mechanical function [1,2]. Mechanisms underlying protection may involve alterations in energy supply (vasodilatation), energy demand (bradycardia, negative inotropy) [3] or energy substrate metabolism (glucose utilization) [1]. While adenosine-induced cardioprotection is pronounced in hearts from healthy animals, results from clinical studies on the benefits of adenosine in diseased hearts have been less dramatic [4]. Similarly, the indirect adenosine mimetic, acadesine (5-aminoimidazole-4-carboxyamide-1-β-D-ribofuranoside, AICAR), an agent that increases adenosine release in ischemic tissue [5], had limited cardioprotective effectiveness in a Phase III trial in patients undergoing coronary artery by-pass graft surgery [6]. The mechanisms underlying the loss of adenosine- and acadesine-induced cardioprotection have not been clarified.

Previously, we observed a loss of the cardioprotective effectiveness of adenosine in hearts that were "stressed" by two transient (10-min) periods of ischemia prior to the onset of severe global ischemia and postulated that adenosine-induced cardioprotection may depend upon the metabolic status of the heart [7]. Although transient ischemia *per se* has no effect on LV mechanical function, it reverses the effects of adenosine on glucose metabolism. Instead of an adenosine-induced inhibition of glycolysis observed in normal hearts [1], adenosine stimulates glycolysis in stressed hearts, an effect that may contribute to acidosis and reduced cardioprotection [7]. As stressing of hearts by transient ischemia may mimic some of the changes observed in the clinical setting, we utilized this

experimental paradigm to determine signaling mechanisms that may account for changes in the metabolic responses to adenosine.

The stress-activated protein kinase, 5'-adenosine monophosphate-activated protein kinase (AMPK), is an important regulator of energy metabolism, and when activated inhibits ATP-consuming biosynthetic pathways while activating ATP-generating catabolic processes [8]. During ischemia-reperfusion, activation of AMPK may be beneficial by increasing ATP production via the stimulation of glucose transport, glyocogenolysis, and glycolysis as well as via the stimulation of fatty acid oxidation through the phosphorylation and inhibition of acetyl-CoA carboxylase (ACC) [9,10,11,12,13]. In contrast, activation of AMPK may be deleterious as stimulation of fatty acid oxidation leads to an inhibition of glucose oxidation (Randle Cycle) [14,15] and this, in combination with elevated rates of glycolysis [16], promotes intracellular acidosis and Na^+ and Ca^{2+} overload that contribute to LV mechanical dysfunction during reperfusion [17].

In this study we investigated whether alterations in AMPK activity underlie changes in the effects of adenosine on energy substrate metabolism in hearts perfused aerobically following stressing by transient ischemia. Our discovery that adenosine caused a marked activation of AMPK under these conditions allowed us to characterize further the consequences of AMPK activation on carbohydrate and fatty acid metabolism under conditions of stable LV mechanical function. Specifically, this study tested the hypothesis that, in accordance with studies on ischemia-induced activation of AMPK, the activation of AMPK in response to adenosine in hearts stressed by transient ischemia also stimulates glycolysis, glucose uptake, and glycogenolysis. In addition, we tested the

hypothesis that activation of AMPK in response to adenosine also accelerates myocardial fatty acid oxidation.

3.2 Materials and Methods

3.2.1 Heart Perfusions

Hearts from sodium pentobarbital-anesthetized male Sprague-Dawley rats (300 to 350 g) that had been fed *ad libitum*, were excised, their aortae were cannulated and a perfusion using Krebs-Henseleit solution (37°C, pH 7.4, gassed with a 95% O_2 - 5% CO_2 mixture) was initiated. Hearts were perfused in the Langendorff mode for 10 min and thereafter switched to the working (ejecting) mode as described previously [1]. The perfusate (recirculating volume of 100 mL) consisted of a modified Krebs-Henseleit solution containing 2.5 mM Ca^{2+}, 11 mM glucose, 1.2 mM palmitate pre-bound to 3% BSA (fatty acid free) and 100 mU/L insulin. Perfusions were performed at a constant workload (preload, 11.5 mm Hg; afterload, 80 mm Hg) and heart rate (paced at 300 beats/min). Heart rate and systolic and diastolic aortic pressures (mm Hg) were measured using a Gould P21 pressure transducer attached to the aortic outflow line. Cardiac output (mL/min) and aortic flow (mL/min) were measured using ultrasonic flow probes (Transonic T206) placed in the left atrial inflow line and the aortic outflow line, respectively. Left ventricular (LV) work (Joules) was calculated as cardiac output x LV developed pressure (systolic pressure - preload pressure) / 1000 x 0.133 and served as a continuous index of LV mechanical function.

3.2.2 Perfusion Protocol

Hearts were perfused under aerobic conditions in working mode for 45 min (Normal Group, no transient ischemia, -TI), or were perfused aerobically for 15 min prior

to stressing by transient ischemia (Stressed Group, two 10-min periods of global no-flow ischemia each followed by 5 min reperfusion, +TI). Hearts were paced at 300 beats/min in all experimental groups, except during the periods of global no-flow ischemia. Normal and Stressed hearts were then either frozen for biochemical analyses (Prior to treatment) or were perfused aerobically for a further 35-min period, either with vehicle (saline) or adenosine (500 μM). Thereafter, untreated and adenosine-treated hearts were frozen for biochemical analyses (End-Treatment) (Fig 3-1A). Two additional groups of Stressed hearts were perfused aerobically during the 35-min treatment period with either phentolamine alone (1 μM), or a combination of phentolamine (1 μM) with adenosine (500 μM).

3.2.3 Immunoblot Analysis of AMPK and ACC

Heart homogenates were obtained by homogenizing frozen LV tissue in a solution containing 20 mM Tris-HCl (pH 7.4 at 4°C), 50 mM NaCl, 50 mM NaF, 5 mM Na pyrophosphate, 0.25 mM sucrose, protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail (Sigma), and 1 mM dithiothrietol. After homogenization for 30 seconds, protein contents of the homogenates were determined using the Bradford protein assay. Samples were diluted and boiled in protein sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose as previously described [18]. Membranes were blocked in 5 % (wt/vol) skim milk powder in Tris Buffered Saline (TBS) containing 0.1% (vol/vol) Tween and then immunoblotted with rabbit anti-phospho-AMPK (Thr172), and rabbit anti-AMPK-total (1:1000 dilution) in 5% BSA (wt/vol) in TBS, or rabbit anti-phospho-ACC (Ser79) (1:1000 dilution) in 5% BSA (wt/vol) in TBS, and peroxidase-conjugated streptavidin (1:500 dilution) in 5%

BSA (wt/vol) in TBS. After extensive washing, the membranes were incubated with a peroxidase-conjugated goat anti-rabbit secondary antibody in 5% skim milk powder (wt/vol) in TBS when appropriate. After further washing, the antibodies were visualized using the Pharmacia Enhanced Chemiluminescence Western Blotting and Detection System. Densitometric analyses of immunoblots (n=3 per experimental group) were performed using Quantity One (4.4.0) Software (Biorad Laboratories). Densitometric values of the phosphorylated proteins were normalized to the total amount of the protein detected and expressed as Arbitrary Density Units (ADU).

3.2.4 Measurement of AMPK Activity

The activity of AMPK (nmol/mg protein/min) was measured in 6% polyethylene glycol (PEG) fractions extracted from 200 mg of frozen LV tissue as described previously [19,20]. Activity of AMPK in the presence of 5'-AMP (200 μM) was assayed in the 6% PEG fraction by following the incorporation of [γ^{32}P] from [γ^{32}P]ATP into a ser-79 phosphorylation site-specific SAMS peptide (HMRSAMSGLHVKRR), as previously described [20,21].

3.2.5 Measurement of Steady State Rates of Palmitate Oxidation

Steady state rates of palmitate oxidation were measured from the release of ^3H_2O, derived from the metabolism of [9,10-^{3}H]palmitate. ^3H_2O was separated from [9,10-^{3}H]palmitate by mixing 0.5 mL of perfusate sampled at predetermined time points (5, 14, 29, 44, 50, 60, 70, 80 min) with 1.88 mL of a 1:2 vol/vol chloroform:methanol solution. Next 1.0 mL of chloroform was added, followed by the addition of 1.0 mL KCl:HCl (1.1 mol/L KCL:0.9 mol/L HCl). Samples were allowed to separate into polar and non-polar phases. A 1.0 ml sample of the polar layer was removed, and mixed with 1.0 mL of

chloroform, 1.0 mL methanol, and 0.9 mL of KCl:HCl solution. Again samples were allowed to separate into polar and non-polar phases, and 0.5 mL aliquots of the polar phase were subjected to scintillation counting for determination of ^3H_2O content. ^3H_2O content of the perfusate, indicative of the metabolism of [9,10-^{3}H]palmitate, was determined in samples removed from the perfusate at 5, 14, 29, 44, 50, 60, 70, 80 min and used to calculate steady state rates of palmitate oxidation for the Baseline and Treatment periods.

3.2.6 Measurement of Adenine Nucleotide Content

Adenine nucleotides were extracted from approximately 100 mg of frozen ventricular tissue into 1 mL 6% ice-cold perchloric acid by homogenization with a pestle in a cold mortar. The tissue perchloric acid mixture was centrifuged at 4ºC, and the supernatant was neutralized with K_2CO_3. High-performance liquid chromatography was used to measure nucleotide and nucleoside content in the neutralized extracts [22].

3.2.7 Measurement of Steady State Rates of Glycolysis and Glucose Oxidation

Glycolysis and glucose oxidation rates were measured directly from the simultaneous production of ^3H_2O (liberated at the enolase step of glycolysis) and $^{14}CO_2$ (liberated at the level of pyruvate dehydrogenase complex and in the citric acid cycle), respectively, from [5-^{3}H]glucose and [U-^{14}C]glucose, as described previously [23,24]. Perfusate was sampled at pre-determined time points (5, 14, 29, 44, 50, 60, 70, 80 min), and steady state rates, expressed as μmol [5-^{3}H]glucose or [U-^{14}C]glucose metabolized/g dry wt/min, were calculated for Baseline and Treatment periods.

3.2.8 Calculation of the Rate of Proton Production Arising from Exogenous Glucose Metabolism

When glucose (from endogenous or exogenous sources) is metabolized by glycolysis and subsequently oxidized 1:1, with the associated synthesis and hydrolysis of ATP, the net production of protons is zero. However, if the rate of glycolysis exceeds that of glucose oxidation, there is a net production of two protons per molecule of exogenous glucose that passes through glycolysis, which is not subsequently metabolized. Therefore the rate of proton production attributable to the hydrolysis of ATP arising from exogenous glucose metabolism can be calculated as 2 X (rate of glycolysis – rate of glucose oxidation).

3.2.9 Assay of Glycogen Content and Glucose Uptake

Frozen LV tissue was powdered using a mortar and pestle maintained at the temperature of liquid N_2. Glycogen, in 200 mg of powdered tissue, was converted to glucose by reacting with 4 M H_2SO_4. The amount of glucose (expressed as μmol glucose units/g dry wt) thus obtained was determined using a Sigma glucose analysis kit. The net rate of glycogen synthesis (μmol glucose/min/g dry wt) in hearts during the 35-min aerobic treatment period was calculated from the increase in [5-^{3}H]- and [^{14}C]glucosyl units in total myocardial glycogen in hearts frozen at End-Treatment relative to hearts frozen Prior to Treatment. The rate of glucose uptake (μmol/min/g dry wt) during the Treatment period was calculated as the sum of the net rate of glycogen synthesis and the rate of glycolysis in individual hearts. The net rate of glycogen degradation was calculated as the difference between the unlabeled myocardial glycogen content in the Prior to Treatment and End-Treatment groups.

3.2.10 Materials

D-[5-^{3}H]glucose, D-[U-^{14}C]glucose, and [9,10-^{3}H]palmitate were purchased form Dupont Canada Inc., ON, Canada. Adenosine was purchased from Research Biochemicals International, Natrick MA, USA. Anti-phospho-AMPK (Thr172), anti-AMPK (total), anti-phospho-ACC antibodies, and peroxidase-conjugated streptavivdin were obtained from Cell Signaling Technology, Beverly, MA, USA. All other chemicals were reagent grade.

3.2.11 Statistical Analysis

Results are expressed as means ± SE (n observations). The significance of differences between untreated and adenosine-treated groups was estimated by Student's *t*-test. When required, multiple comparisons were made using one-way analysis of variance (ANOVA). When ANOVA revealed significant differences, individual groups were compared using Bonferroni's multiple comparisons test. Differences were considered significant when $P < 0.05$.

3.3 Results

3.3.1 Left Ventricular Work in Normal and Stressed Hearts

LV work was stable and similar in normal and stressed hearts prior to being assigned to the vehicle- or adenosine-treated groups. While all measurable LV work ceased during the periods of transient ischemia, it recovered quickly and remained stable throughout subsequent perfusion in the absence (vehicle-treated) or presence of adenosine. Adenosine had no effect on LV work in normal or stressed hearts (Fig. 3-1B). Coronary flow and coronary vascular conductance were increased by adenosine ($P<0.05$) in normal, but not in stressed hearts (Table 3-1).

3.3.2 Phosphorylation of AMPK and ACC in Normal and Stressed Hearts

To determine the consequences of transient ischemia *per se*, the phosphorylation of AMPK and its downstream target ACC were assessed in normal and stressed hearts frozen prior to the treatment period. There was no difference in AMPK (Fig. 3-2A) or ACC (Fig. 3-2B) phosphorylation between these groups. Thus, data presented hereafter pertain specifically to alterations in AMPK phosphorylation and cardiac energy metabolism in response to adenosine treatment.

3.3.3 Effects of Adenosine on the Phosphorylation of AMPK and ACC

Adenosine decreased AMPK phosphorylation by 70% and activity by 50% in normal hearts (unpaired *t*-test, $P<0.05$) consistent with our previous reports using N^6-cyclohexyladenosine (CHA), an adenosine A_1 receptor agonist [24]. Adenosine increased AMPK phosphorylation (9-fold, Fig. 3-3A) and activity (2.3-fold, Fig. 3-3B) in stressed hearts ($P<0.05$). Relative to adenosine-treated normal hearts, AMPK phosphorylation and activity were 30-fold and 6-fold higher, respectively, in adenosine-treated stressed hearts (Figs. 3-3A and 3-3B).

The short term regulation of fatty acid oxidation is, in part, achieved via the reversible phosphorylation and inactivation of ACC. Adenosine inhibited the phosphorylation of both ACCβ and ACCα in normal hearts (Fig. 3-4A); however, as we have reported previously [25], it did not affect rates of palmitate oxidation (Fig. 3-4B). Interestingly, despite the marked adenosine-induced activation of AMPK in stressed hearts, adenosine did not increase the phosphorylation of either ACCβ or ACCα, and did not accelerate rates of palmitate oxidation.

3.3.4 Effects of Adenosine on Adenine Nucleotide Content

As an increase in AMP concentration and/or a decrease in the ATP/AMP ratio regulate AMPK activity, the contents of these adenine nucleotides were assessed in vehicle- and adenosine-treated hearts. While adenosine did not affect ATP content in stressed hearts (Fig. 3-5A), it increased AMP content 1.6-fold ($P<0.05$) (Fig. 3-5B) and consequently decreased the ratio of ATP/AMP by 40% ($P<0.05$) (Fig. 3-5C), thereby mimicking changes expected to occur with a decrease in cellular energy state. Although adenosine increased AMP content of normal hearts, it also increased ATP content, and so caused no change in the ATP/AMP ratio (Table 3-2).

3.3.5 Effects of AMPK Activation on Glucose Metabolism

Steady-state rates of glucose metabolism (glycolysis and glucose oxidation) and calculated rates of proton production were assessed to characterize the consequences of the adenosine-induced activation of AMPK in stressed hearts. In hearts with high AMPK activity, both the rates of glycolysis (1.8-fold, $P<0.05$) and glucose oxidation (1.8-fold, $P<0.05$) were accelerated (Fig. 3-6A & 3-6B). Consequently, the calculated rate of proton production from exogenous glucose was significantly greater (1.7-fold, $P<0.05$) in hearts with elevated AMPK activity (Fig. 3-6C).

3.3.6 Effects of AMPK Activation on Glucose Uptake and Glycogen Metabolism

Rates of glucose uptake, as well as glycogen synthesis and degradation were also calculated in hearts perfused in the absence and presence of adenosine. Despite the marked activation of AMPK in adenosine-treated hearts, the rate of glucose uptake was not different from that calculated in vehicle-treated hearts (Fig. 3-7A). As an increase in glucose uptake was not responsible for the elevated rate of glycolysis observed in hearts

with high AMPK activity, glycogen metabolism (glycogen synthesis and glycogen degradation) was assessed. In vehicle-treated hearts, the net rate of glycogen synthesis was approximately 22% of the rate of glucose uptake. In hearts with high AMPK activity, glycogen synthesis was completely suppressed ($P<0.05$) (Fig. 3-7B), and consequently the rates of glucose uptake (Fig. 3-7A) and glycolysis (Fig. 3-6A) were equivalent. The net rate of glycogenolysis was not different between hearts with low and high AMPK activity (Fig. 3-7C). Inhibition of glycogen synthesis and unaltered glycogen degradation in hearts with high AMPK activity resulted in a slight, but not statistically significant, decrease in glycogen content at the end of the treatment period in adenosine-treated hearts (Fig. 3-7D). Overall, these data suggest that it is an alteration in glucose utilization, i.e. the partitioning of glucose between glycogen synthesis and glycolysis following uptake, rather than an increase in glucose uptake that accounts for the acceleration of glycolysis in hearts with high AMPK activity.

3.3.7 Effects of AMPK Activation on Steady-State Rates of ATP Production

The activation of AMPK by adenosine in stressed hearts significantly increased calculated rates (μmol/g dry wt/min) of ATP production from glycolysis (6.1±1.1 to 10.9±0.9, $P<0.05$) and glucose oxidation (21.1±2.6 to 35.5±4.9, $P<0.05$), but had no significant effect on the calculated rate of ATP production from palmitate oxidation (115.5±19.4 to 133.8±21). As palmitate oxidation is the major energy source, overall ATP production was not increased in hearts with high AMPK activity.

3.3.8 Effects of Phentolamine on AMPK Phosphorylation

We have previously demonstrated that phentolamine (1 μM), an α-adrenoceptor antagonist, prevents the adenosine-induced acceleration of glycolysis and the calculated

rate of proton production in hearts stressed by transient ischemia [26]. Examination of the effects of phentolamine on the phosphorylation of AMPK showed that while it has no effect on AMPK phosphorylation *per se*, it prevented the adenosine-induced increase in AMPK phosphorylation in stressed hearts (Fig. 3-8).

3.4 Discussion

3.4.1 Summary of Major Findings

This study investigated the role of AMPK in mediating the effects of adenosine on myocardial energy metabolism. During aerobic perfusion of hearts stressed by transient ischemia and under conditions of stable mechanical function, adenosine stimulated AMPK activity. Despite the marked increase in AMPK activity, ACC phosphorylation and palmitate oxidation were not affected. However, increases in both glycolysis and glucose oxidation were observed. As glucose uptake in hearts with high AMPK activity was unchanged, the observed increase in glucose metabolism arose from an altered partitioning of glucose following its uptake away from glycogen synthesis to glycolysis and glucose oxidation. This indicates that in contrast to the ischemia-induced activation of AMPK, adenosine-induced activation of AMPK under conditions of stable LV work is not sufficient to stimulate fatty acid oxidation or glucose uptake. Thus, previous associations between high AMPK activity and elevated rates of fatty acid oxidation and glucose uptake in ischemic tissue may have arisen as a consequence of other ischemia-induced changes rather than to a direct activation of AMPK.

3.4.2 Transient Ischemia – A Unique Model to Characterize the Involvement of AMPK in the Regulation of Myocardial Glucose Metabolism

These studies were performed using isolated working rat hearts, a well-established model for the simultaneous and direct measurement of cardiac mechanical and metabolic function. The inclusion of both glucose and palmitate as energy substrates in the perfusate ensured that myocardial glycogen content was not depleted, thereby preserving normal values of glycogen turnover [7,27]. The presence of insulin ensured adequate glucose transport, so that glucose availability was not rate limiting for glycolysis and glucose oxidation. Furthermore, the concentrations of energy substrates provided in the perfusate mimic some of the metabolic conditions that occur following myocardial ischemia. The periods of transient ischemia that caused alterations in the responses to adenosine do not induce ischemic preconditioning as recovery of LV mechanical function following a period of sustained severe ischemia is unaltered [7]. Also, as transient ischemia is not of sufficient duration to cause LV mechanical dysfunction during the treatment periods, this experimental model provided the opportunity to characterize the role of AMPK in mediating adenosine-induced alterations in energy substrate metabolism under aerobic conditions of stable energy supply and demand.

3.4.3 Non-ischemic Activation of AMPK

Examination of AMPK phosphorylation status (indicative of activity) and direct assays of AMPK activity provide clear evidence that, relative to vehicle-treated hearts, adenosine increased AMPK activity in hearts stressed by transient ischemia. Activation of AMPK was not due to transient ischemia *per se* as AMPK phosphorylation following transient ischemia, but prior to adenosine treatment, was identical to that seen in normal

hearts. The mechanism underlying the activation of AMPK by adenosine is unlikely to be mediated by adenosine A_1 receptors, as the A_1 receptor agonist N^6-cyclohexyladenosine inhibits AMPK activation in isolated working rat hearts during low-flow ischemia as well as after transient ischemia [23,24]. Adenosine A_2 receptor stimulation is also not likely involved as the selective adenosine A_2 receptor agonist, CGS21680 inhibits AMPK activation [28]. Stimulation of α-adrenoceptors activates AMPK in Chinese hamster ovary cells [29], and we have previously implicated α-adrenoceptors in adenosine-induced stimulation of glycolysis following transient ischemia [26]. In this study we demonstrate that the α-adrenoceptror antagonist phentolamine, which prevents the adenosine-induced stimulation of glycolysis following transient ischemia [26], also abrogates the phosphorylation of AMPK in response to adenosine in stressed hearts. This suggests that activation of α-adrenoceptors plays a role in the adenosine-induced activation of AMPK and supports the observation that the associated downstream alterations in myocardial glucose metabolism were due to AMPK activation.

3.4.4 Mechanisms Underlying the Non-ischemic Activation of AMPK

Although the use of AICAR is precluded in this study due to its marked depressant effects on LV function at concentrations required to enhance AMPK activity, previous studies have used AICAR to activate AMPK independent of ischemia, possibly by mimicking ischemic conditions by decreasing the ATP/AMP ratio. The activation of AMPK by adenosine in hearts stressed by transient ischemia may involve a similar mechanism. Although tissue levels of phospocreatine (PCr), creatine (Cr) and the PCr/Cr ratio (Table 3-3), as well as ATP content were not significantly different between groups at the end of the treatment period, the higher AMP content in adenosine-treated hearts, as

well as the decreased ATP/AMP ratio suggests that, in the isolated perfused rat heart, AMP is capable of surmounting ATP-mediated inhibition of AMPK activity. Although the measurement of total AMP content by high pressure liquid chromatography in this study may overestimate cytosolic [AMP], as most AMP is bound, our findings are consistent with a previous report suggesting that ATP inhibition of AMPK activity can be overcome by a small change (~ 1.8 μM) in the free cytosolic concentration of AMP [30]. The elevated AMP content in hearts treated with adenosine following transient ischemia (Fig. 3-5B) may be attributed to an increased influx of adenosine from the vascular compartment into the cardiac myocyte and subsequent phosphorylation by adenosine kinase [31]. This is evinced by the increase in total tissue content of adenosine (data not shown) as well as AMP. AMPK activation in response to a significant change in the ATP/AMP ratio may rule out a role for Ca^{2+}/calmodulin-dependent protein kinase kinases (CaMKKs) as AMPK-activating kinases under these conditions, as CaMKKs appear to activate AMPK independent of changes in the ATP/AMP ratio, and are not influenced by AICAR [32].

3.4.5 Activation of AMPK does not Stimulate Myocardial Palmitate Oxidation

Numerous reports have linked AMPK activation to the stimulation of fatty acid oxidation. AMPK activation has been associated with enhanced clearance of long chain fatty acid (LCFA) in cardiac muscle preparations *in vivo* [33], and with increased rates of myocardial fatty acid oxidation during post-ischemic reperfusion [19]. However, it was not clearly defined whether enhanced fatty acid utilization was due solely to AMPK activation, and not to other changes that may have occurred as a consequence of ischemia and altered LV mechanical function. The lack of association between AMPK activation

and a stimulation of the rate of palmitate oxidation observed in this study indicates that AMPK activation is not sufficient to stimulate fatty acid oxidation in the aerobically perfused working rat heart. Moreover, while the underlying mechanism for the stimulation of fatty acid oxidation by AMPK has been considered to result from AMPK-induced phosphorylation and inactivation of ACC, the extent of ACC (ACCα and ACCβ) phosphorylation was unaltered in the face of markedly elevated AMPK activity. Furthermore, although the phosphorylation of ACC is attenuated in response to adenosine in normal hearts (absence of transient ischemia), there is no associated change in palmitate oxidation. These observations differ from previous reports that suggested a negative correlation between AMPK and ACC activity, coupled to increased fatty acid oxidation [11,12,19,34,35]. However, these differences may arise due to differences in experimental models (skeletal versus cardiac muscle, severity of myocardial ischemia). Moreover, the dissociation between AMPK and ACC phosphorylation in this study is consistent with a recent report indicating a lack of ACC phosphorylation despite AMPK activation in rat heart following mild ischemia [36].

3.4.6 Activation of AMPK Uncouples Glycolysis and Glucose Oxidation

Following entry into the cardiac myocyte, about 80% of glucose enters the glycolytic pathway which generates a limited amount of ATP (2 moles per mole of glucose), and pyruvate for subsequent oxidation. The observation that glycolysis was accelerated in hearts with high AMPK activity is compatible with previous observations that indicate AMPK stimulates the phosphorylation and activation of 6-phosphofructo-2 kinase, an enzyme that generates fructose 2,6 bisphosphate, a potent stimulator of 6-phosphofructo-1 kinase, the rate-limiting step of glycolysis [10,37]. Glucose oxidation, the

final stage of glucose metabolism where pyruvate undergoes oxidative decarboxylation by pyruvate dehydrogenase to generate acetyl-CoA for the tricarboxylic acid cycle, was also accelerated by adenosine. Although previous studies suggest that increasing glycolysis during ischemia and reperfusion plays a protective role in limiting myocardial damage [13], the associated increase in the calculated rate of proton production and potential for Na^+ and Ca^{2+} overload [17] may explain the loss of cardioprotective effectiveness of adenosine in stressed hearts as reported previously [7].

3.4.7 Activation of AMPK does not Affect Glucose Uptake, but Alters Glycogen Metabolism

The ability to activate AMPK in the absence of confounding factors that occur a) during severe ischemia, b) with alterations in mechanical function and energy demand, or c) in isolated cell systems (low energy demand) provided a unique opportunity to characterize the relationships between AMPK activity and the pathways of glucose uptake and glycogen metabolism under conditions of stable LV work. Acceleration of myocardial glucose uptake by various stresses is independent of insulin signaling and may be due to the activation of AMPK [38,39]. Indeed, activation of AMPK by metabolic stresses in isolated skeletal muscle, or pharmacologically in isolated heart muscle by AICAR, stimulates glucose uptake, which may result from an AMPK-mediated translocation of GLUT4 transporters from an intracellular membrane pool to the cell surface [39,40,41]. Whether activation of AMPK activity alone is sufficient to increase glucose uptake in the absence of ischemia or changes in energy demand has not been clearly defined. However, this study has shown that, in spite of marked increases in AMPK activity, glucose uptake was not markedly affected. The low statistical power of

this comparison may have concealed a significant increase, but the observed 15% difference was small relative to the more than 2-fold increase in glucose uptake that can be achieved in the absence of palmitate [18]. That result also indicates that the lack of effect of AMPK activation on glucose uptake was not due to maximal rates of uptake pre-existing in the working heart. Thus it appears that AMPK activation is not sufficient to stimulate glucose uptake in working cardiac muscle in the absence of other stimuli that may occur concomitantly with ischemia. This finding is in agreement with a recent study that demonstrated that AMPK activation does not affect cardiac glucose clearance *in vivo* [33]. Furthermore, in contrast to previous reports linking increased glucose uptake and elevated rates of glycolysis [10,37,42], the present study indicates that increased glucose uptake does not underlie the acceleration of glycolysis in response to the adenosine-induced activation of AMPK, suggesting that these two processes are differentially regulated under conditions of stable LV work.

Glycogen is an important source of endogenous glucose, and its turnover (simultaneous synthesis and degradation) is a key factor in the control of glucose utilization [24,27]. Glycogen accumulates to physiological levels (150-160 μmol/g dry wt) in aerobic working rat hearts perfused with glucose, insulin, and palmitate, and, although there is continuous glycogen turnover, the net rate of glycogenolysis is negligible [27]. The demonstration that net glycogen synthesis was depressed in hearts with high AMPK activity, while glycogenolysis was unaffected, reflects an altered partitioning of glucose utilization following uptake in hearts with elevated AMPK activity. This may arise due to suppression of glycogen synthase activity [43,44] thereby directing glucose away from

energy storage (glycogen synthesis) or by an activation of glycolysis thereby diverting glucose towards energy production (glycolysis and glucose oxidation).

3.4.8 Conclusions

In conclusion, this study has shown that the activation of AMPK by adenosine during aerobic perfusion, and under conditions of stable energy supply and demand, does not alter overall rates of energy production. Rather, activation of AMPK stimulates glycolysis and glucose oxidation and accelerates the calculated rate of proton production, but has no effect on rates of glucose uptake and palmitate oxidation. This indicates that AMPK regulates myocardial glucose utilization in a coordinated manner by influencing the relative rates of glycolysis and glycogen synthesis. As AMPK activation increases the calculated rate of proton production and the potential for acidosis, inhibition of AMPK may be a useful approach to restore adenosine-induced cardioprotection in stressed hearts.

Figure 3-1. Experimental protocol for heart perfusions. A, Hearts were either perfused under aerobic conditions in working mode for 45 min (Normal Group, no transient ischemia, -TI), or were perfused aerobically for 15 min prior to stressing by transient ischemia (+TI, two 10-min periods of global no-flow ischemia (shaded bars) each followed by 5 min reperfusion, Stressed Group). normal and stressed hearts were then either frozen for biochemical analyses (Prior to treatment, ↓) or were assigned randomly to either a vehicle (saline) treated group (-TI, n=9, +TI, n=10) or to a group that was treated with adenosine (500 μM) (-TI, n=9, +TI, n=7) and perfused aerobically for a further 35-min period. Hearts were then frozen for biochemical measurements (End-Treatment, ↓). B, Mechanical function (LV work, mean ± SE) of normal (vehicle, □ ; adenosine, ■) and stressed (vehicle, ○ ; adenosine, ●) hearts.

Figure 3-1

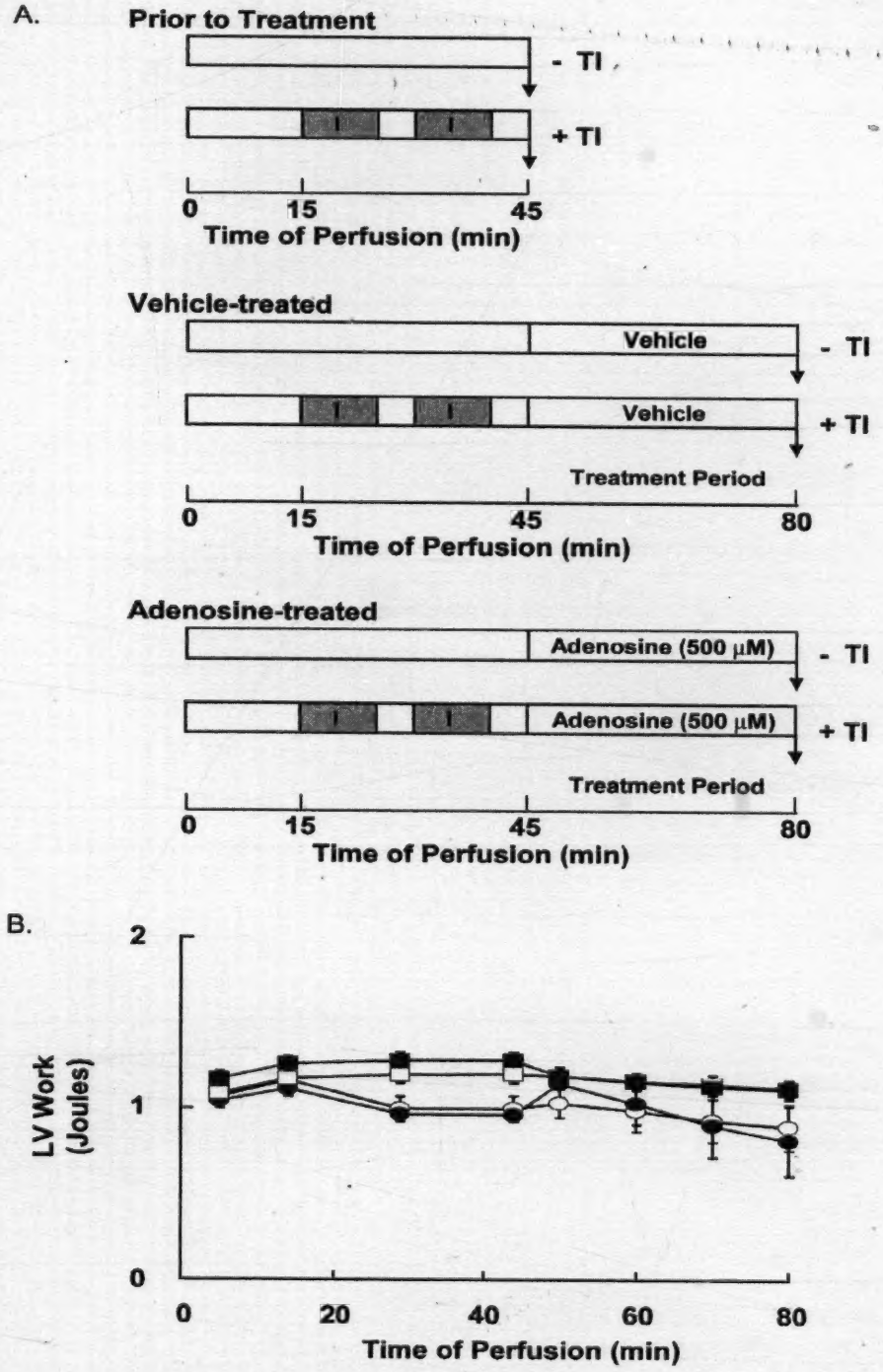
A.
Prior to Treatment
- TI
+ TI
0
15
45
Time of Perfusion (min)
Vehicle-treated
Vehicle
- TI
Vehicle
+ TI
Treatment Period
0
15
45
80
Time of Perfusion (min)
Adenosine-treated
Adenosine (500 μM)
- TI
Adenosine (500 μM)
+ TI
Treatment Period
0
15
45
80
Time of Perfusion (min)
B.
LV Work (Joules)
2
1
0
0
20
40
60
80
Time of Perfusion (min)

Figure 3-2. Phosphorylation of AMPK and ACC prior to treatment. Immunoblots and their densitometric analysis were performed as described under "Materials and Methods". A, *Top panel,* representative immunoblots of phosphorylated AMPK and total AMPK from ventricular homogenates of normal (-TI) and stressed (+TI) hearts. *Bottom panel,* densitometric analysis of immunoblots of normal (-TI, n=3, open bars) and stressed (+TI, n=3, black bars) hearts. B, *Top panel,* representative immunoblots of phosphorylated ACC and total ACC from ventricular homogenates of normal (-TI) and stressed (+TI) hearts. *Bottom panel,* densitometric analysis of immunoblots of normal (-TI, n=3, open bars) and stressed (+TI, n=3, black bars) hearts. Values represent means ± SE.

Figure 3-2

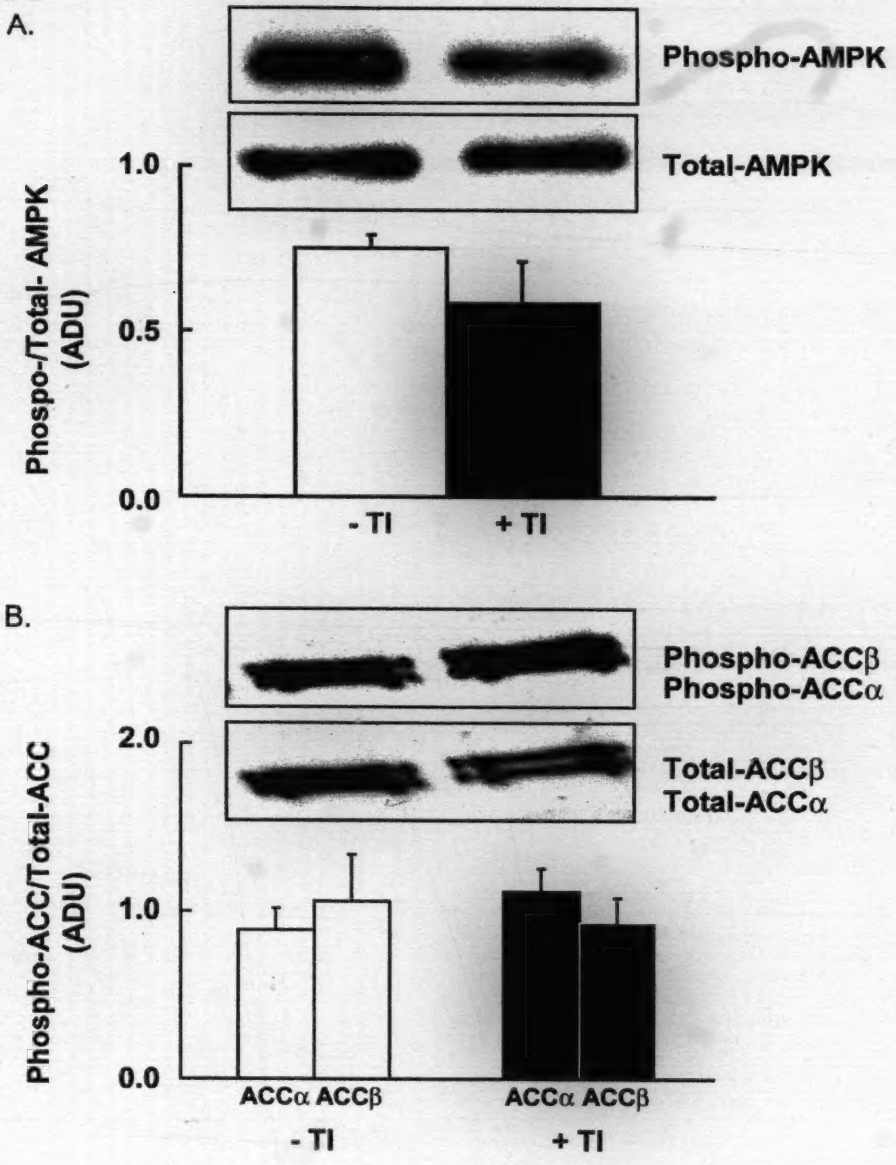
A.
Phospho-AMPK
Total-AMPK
Phospo-/Total- AMPK (ADU)
1.0
0.5
0.0
- TI
+ TI
B.
Phospho-ACCβ
Phospho-ACCα
Total-ACCβ
Total-ACCα
Phospho-ACC/Total-ACC (ADU)
2.0
1.0
0.0
ACCα ACCβ
ACCα ACCβ
- TI
+ TI

Figure 3-3. AMPK phosphorylation and AMPK activity in vehicle- and adenosine-treated normal and stressed hearts. Immunoblots, their densitometric analysis (ADU) and AMPK activity (nmol/mg protein/min) were measured at End-Treatment as described under "Materials and Methods." A, *Top panel,* representative immunoblots of phosphorylated AMPK and total AMPK from ventricular homogenates. *Bottom panel,* densitometric analysis of immunoblots (n=3 per group). B, AMPK activity in PEG fractions extracted from ventricular tissue (n=3 per group). Values represent means ± SE and are shown for normal (-TI) and stressed (+TI) hearts perfused in the absence (-) or presence (+) of adenosine (Ado). * indicates a significant difference from vehicle-treated stressed hearts; †indicates a significant difference from adenosine-treated normal hearts.

Figure 3-3

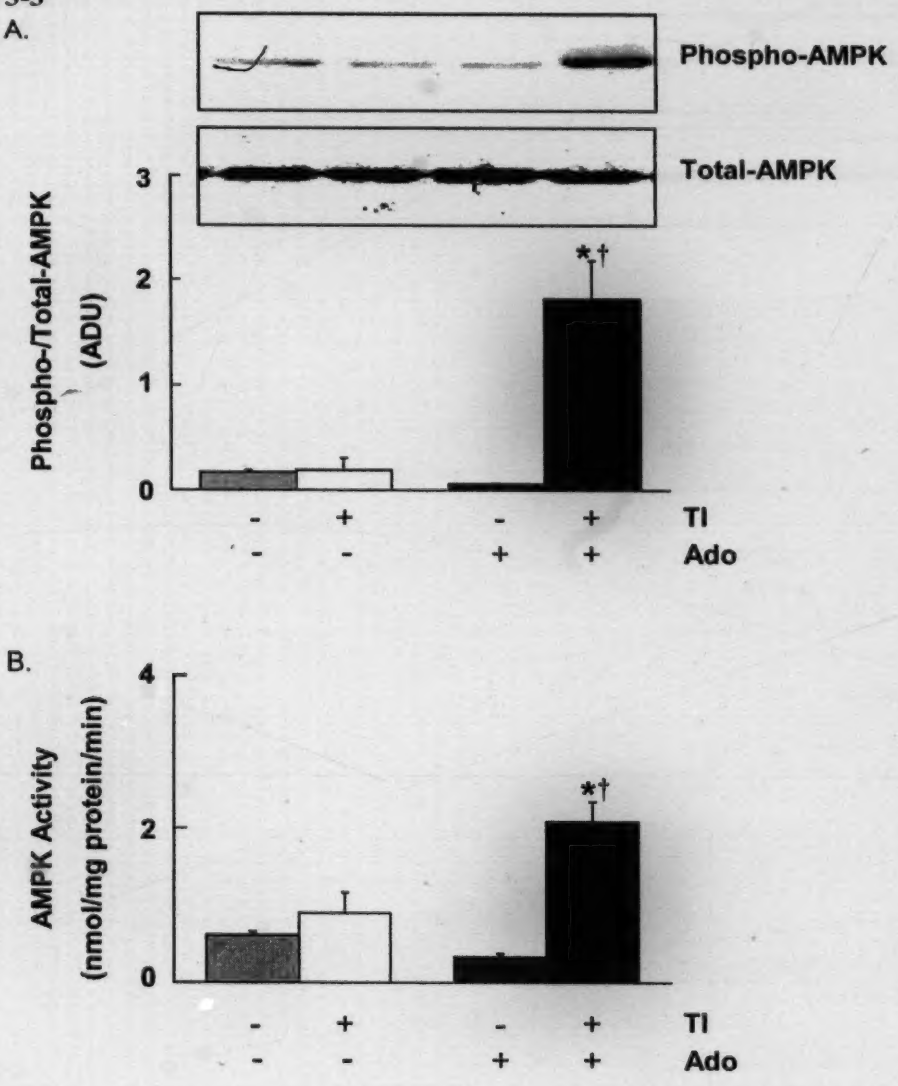
A.
Phospho-AMPK
Total-AMPK
Phospho-/Total-AMPK (ADU)
3
2
1
0
*†
- + - + TI
- - + + Ado
B.
AMPK Activity (nmol/mg protein/min)
4
2
0
*†
- + - + TI
- - + + Ado

Figure 3-4. ACC phosphorylation and palmitate oxidation in vehicle- and adenosine-treated normal and stressed hearts. Immunoblots, their densitometric analysis (ADU) and palmitate oxidation (μmol/g dry wt/min) were measured as described under "Materials and Methods." *A, Top panel,* representative immunoblots of phosphorylated ACCα and ACCβ from ventricular homogenates. *Bottom panels,* densitometric analysis of immunoblots (n=3 per group) for normal (-TI) and stressed (+TI) hearts perfused in the absence (-) or presence (+) of adenosine (Ado). B, Palmitate oxidation measured during the aerobic treatment period for normal hearts (-TI) perfused in the absence (-, n=6) or presence (+, n=7) of adenosine (Ado) and for stressed hearts (+TI) perfused in the absence (-, n=7) or presence (+, n=5) of adenosine (Ado). Values represent means ± SE. * indicates a significant difference from vehicle-treated stressed hearts.

Figure 3-4

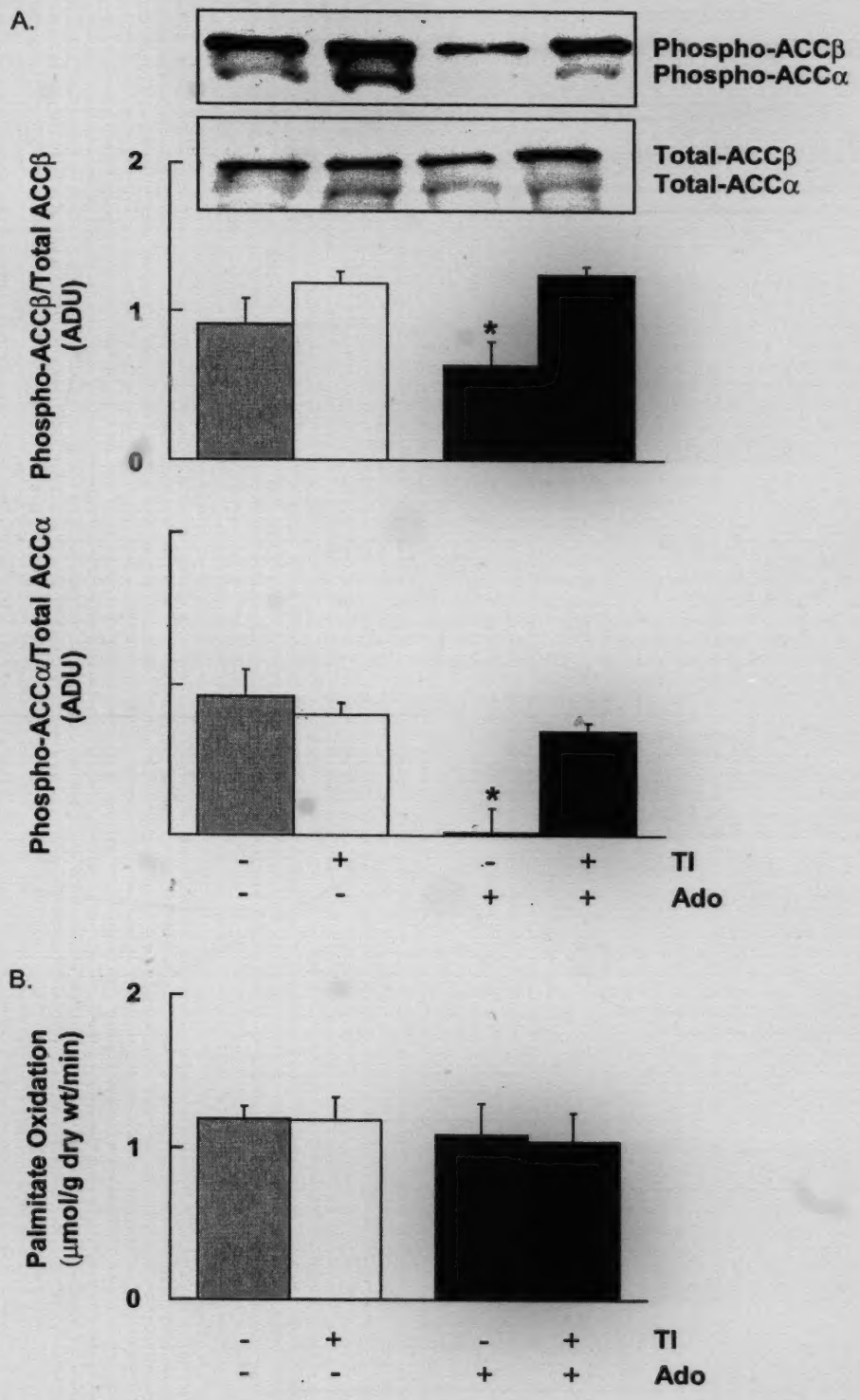
A.
Phospho-ACCβ
Phospho-ACCα
Total-ACCβ
Total-ACCα
Phospho-ACCβ/Total ACCβ (ADU)
Phospho-ACCα/Total ACCα (ADU)
2
1
0
*
*
- + - + TI
- - + + Ado
B.
Palmitate Oxidation (μmol/g dry wt/min)
2
1
0
- + - + TI
- - + + Ado

Figure 3-5. Adenine nucleotide content in stressed hearts. ATP (A) and AMP (B) content (μmol/g dry wt) and the ATP/AMP ratio (C) in hearts frozen at End-Treatment was measured as described under "Materials and Methods". Open bars represent vehicle-treated hearts (n=8) and black bars represent adenosine-treated hearts (n=6). Values represent means ± SE. * indicates as significant difference from vehicle-treated hearts.

Figure 3-5

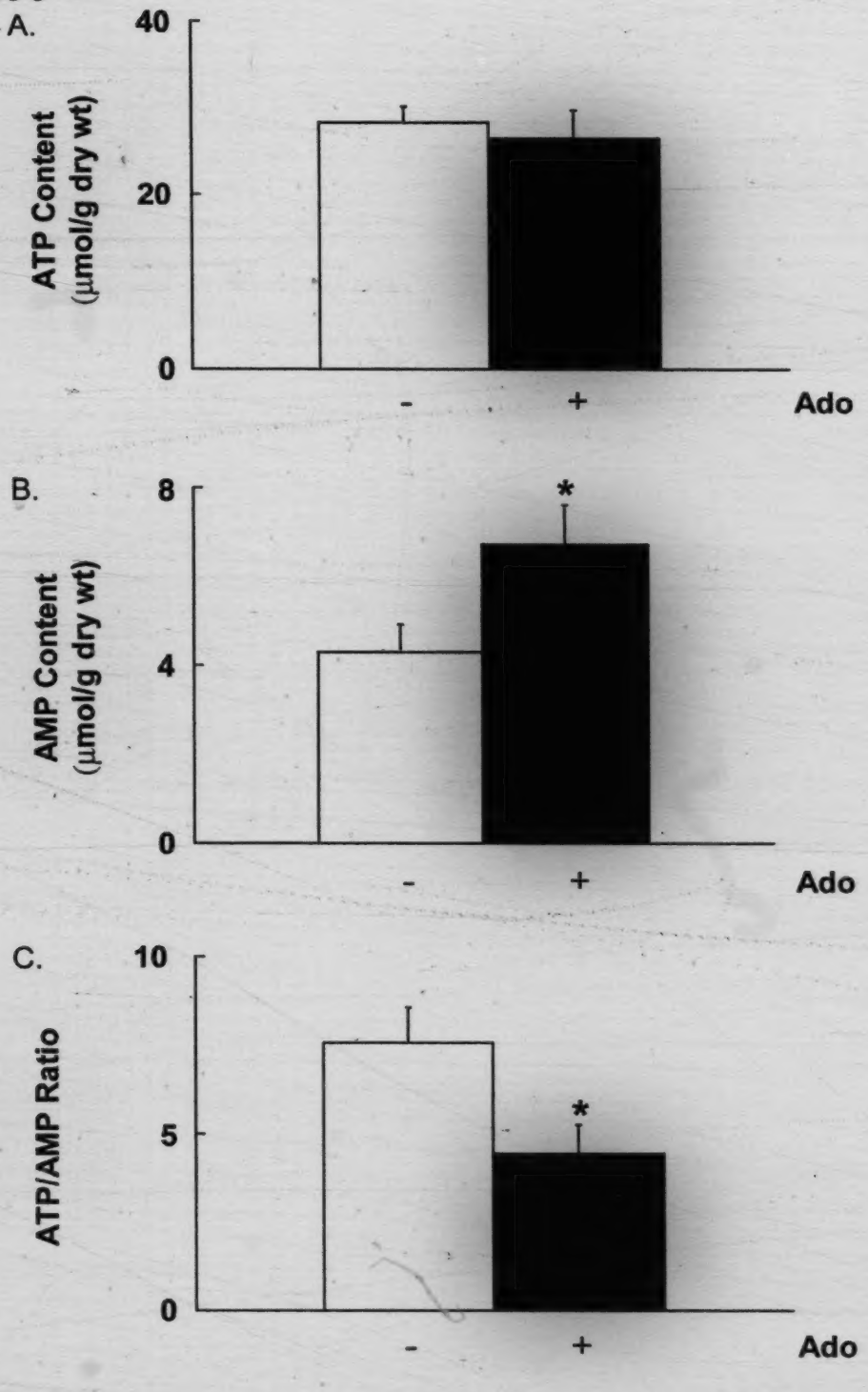
A.
40
20
0
ATP Content
(μmol/g dry wt)
-
+
Ado
B.
8
4
0
*
AMP Content
(μmol/g dry wt)
-
+
Ado
C.
10
5
0
*
ATP/AMP Ratio
-
+
Ado

Figure 3-6. Rates of glucose metabolism and calculated proton production in stressed hearts. Glycolysis (A), glucose oxidation (B) and the calculated rate of proton production from glucose metabolism (C) were assessed as described under "Materials and Methods." Open bars represent vehicle-treated hearts (n=10) and black bars represent adenosine-treated hearts (n=7). Values (μmol/g dry wt/min) represent means ± SE. * indicates a significant difference from vehicle-treated hearts.

Figure 3-6

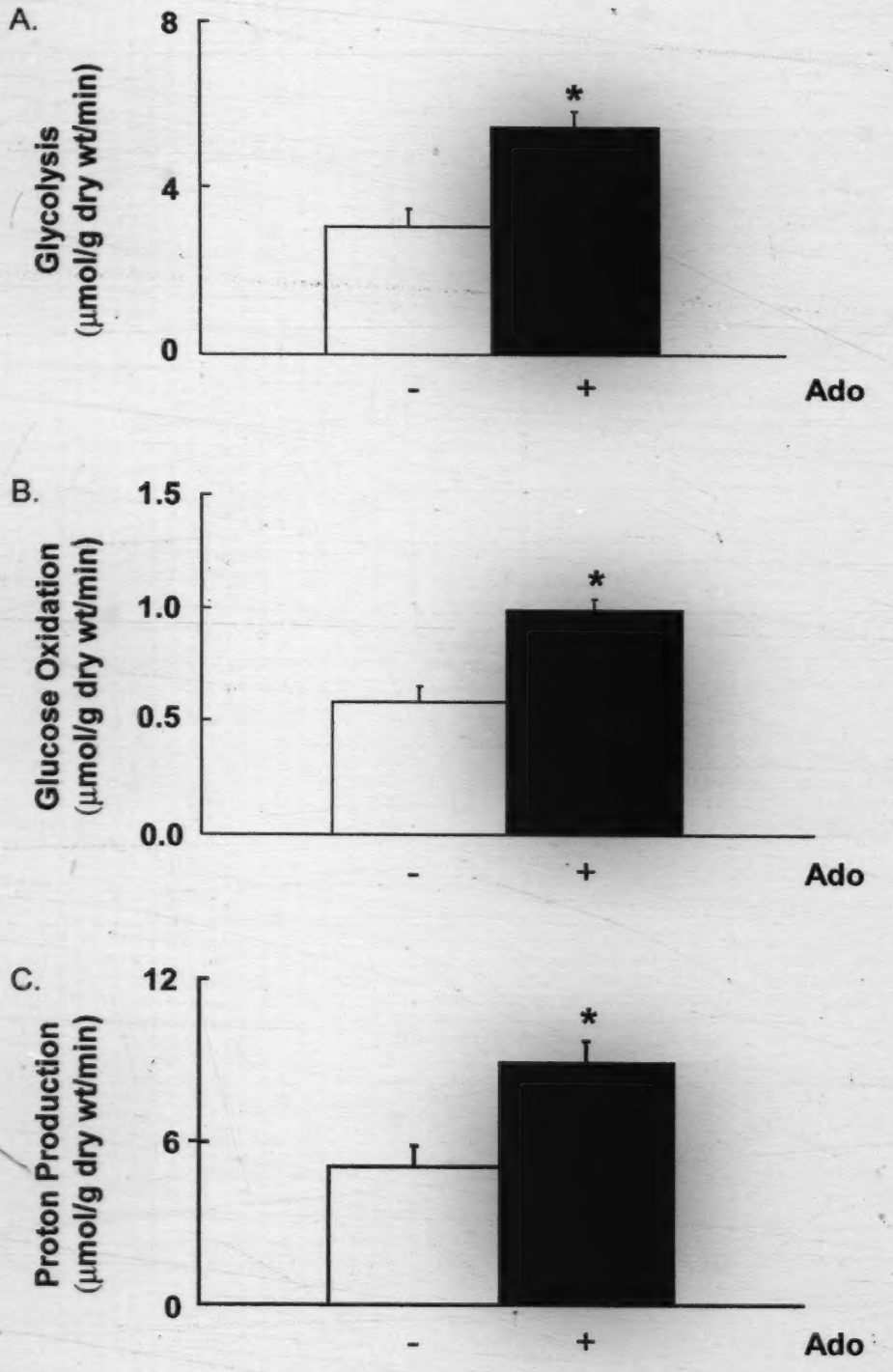
A.
Glycolysis
(μmol/g dry wt/min)
8
4
0
*
-
+
Ado
B.
Glucose Oxidation
(μmol/g dry wt/min)
1.5
1.0
0.5
0.0
*
-
+
Ado
C.
Proton Production
(μmol/g dry wt/min)
12
6
0
*
-
+
Ado

Figure 3-7. Glucose uptake and glycogen metabolism in stressed hearts. Glucose uptake (A), glycogen synthesis (B), glycogen degradation (C) and glycogen content (D) were assessed as described under "Materials and Methods." Open bars represent vehicle-hearts (n=6) and black bars represent adenosine-treated hearts (n=6). Values (μmol/g dry wt/min for A ,B, and C and μmol/g dry wt for D) represent means ± SE. * indicates a significant difference from vehicle-treated hearts.

Figure 3-7

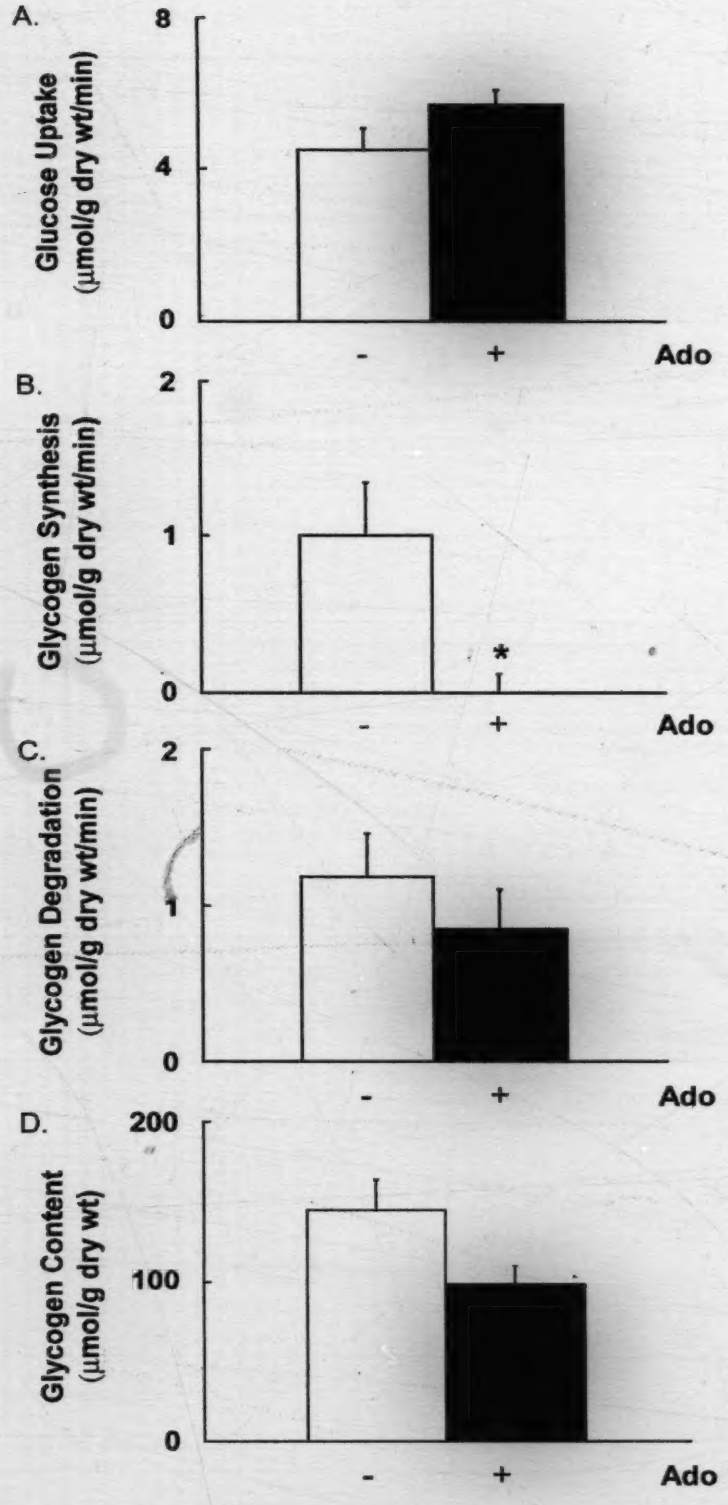
A.
Glucose Uptake
(μmol/g dry wt/min)
8
4
0
-
+
Ado
B.
Glycogen Synthesis
(μmol/g dry wt/min)
2
1
0
*
-
+
Ado
C.
Glycogen Degradation
(μmol/g dry wt/min)
2
1
0
-
+
Ado
D.
Glycogen Content
(μmol/g dry wt)
200
100
0
-
+
Ado

Figure 3-8. Effects of phentolamine on AMPK phosphorylation in stressed hearts. Immunoblots for phospho- and total AMPK and their densitometric analysis (ADU) were performed as described under "Materials and Methods." Values represent means ± SE (n=3 per group) and are shown for stressed (+TI) hearts treated with vehicle perfused in the absence (-) or presence (+) of phentolamine and for stressed (+TI) hearts perfused with adenosine (Ado) in the absence (-) or presence (+) of phentolamine. * indicates a significant difference from hearts perfused in absence of phentolamine, † indicates significant difference from hearts perfused in the presence of adenosine.

Figure 3-8

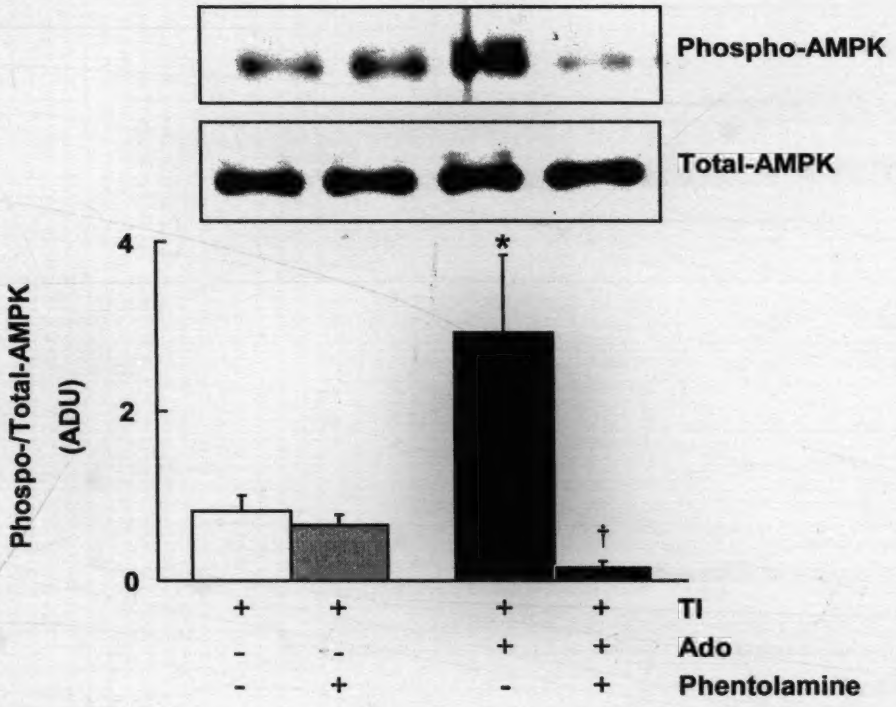
Phospho-AMPK
Total-AMPK
Phospo-/Total-AMPK (ADU)
4
2
0
*
†
+ + + + TI
- - + + Ado
- + - + Phentolamine

Table 3-1. Cardiac function in normal and stressed hearts.

Cardiac Parameter	Vehicle		Adenosine	
	-TI	+TI	-TI	+TI
HR (beats/min)	300	300	300	300
RPP (HR x PSP x 10^{-3})	38.3 ± 0.7	35.2 ± 1.8	35.7 ± 0.3	36.1 ± 1.6
AF (mL/min)	46.5 ± 2.2	38.9 ± 5.1	38.9 ± 2.2	36.7 ± 6.8
CF (mL/min)	31.3 ± 2.7	26.1 ± 2.6	40.4 ± 2.0*†	27.1 ± 1.9
CVC (mL/min/mmHg)	0.332 ± 0.028	0.300 ± 0.025	0.449 ± 0.022*†	0.300 ± 0.020

Functional parameters were measured in vehicle-treated normal (-TI, n=9) and stressed hearts (+TI, n=10) and adenosine-treated normal (-TI, n=7) and stressed hearts (+TI, n=7). Values represent means ± SE for heart rate (HR), rate pressure product (RPP), aortic flow (AF), coronary flow (CF), and coronary vascular conductance (CVC). * indicates a significant difference from vehicle-treated normal hearts, † indicates significant difference from adenosine-treated stressed hearts.

Table 3-2. End-Treatment values of adenosine and adenine nucleotide content in ventricular tissue from normal hearts.

Adenine Nucleoside/Nucleotide	Vehicle	Adenosine
Ado	0.6±0.2	1.5±0.3*
AMP	3.8±0.5	4.3±0.5
ATP	29.4±1.6	34.9±0.7*
ATP/AMP	5.4±1.8	5.7±1.9

Adenosine (Ado) and adenine nucleotide contents (μmol/g dry wt) were measured as described under "Materials and Mehtods" in vehicle-treated (n=6) and adenosine-treated (n=6) normal hearts frozen at the end of the treatment period. Values represent means ± SE. * indicates a significant difference from vehicle-treated hearts.

Table 3-3. End-Treatment values of PCr and Cr content in ventricular tissue.

High Energy Phosphate	Vehicle	Adenosine
PCr	62.2±5.1	59.7±4.7
Cr	194.7±5.1	194.0±10.6
PCr/Cr	0.32±0.03	0.31±0.08

PCr and Cr contents (μmol/g dry wt) were measured as described under "Materials and Methods" in vehicle-treated (n=8) and adenosine-treated (n=6) stressed hearts frozen at the end of the treatment period. Values represent means ± SE. There were no significant differences between groups.

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Chapter 4

p38 Mitogen Activated Protein Kinase Mediates Adenosine-Induced Alterations in Myocadial Glucose Utilization Via 5'-AMP Activated Protein Kinase

HPLC analysis of adenosine and adenine nucleotides was performed by Ken Strynadka.

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A version of this chapter has been published in the *American Journal of Physiology (Heart Circ Physiol).* Jaswal JS, Gandhi M, Finegan BA, Dyck JRB, and Clanachan AS. Epub ahead of print, Dec. 15, 2006.

4.1 Introduction

Cardioprotection induced by adenosine and the adenosine A_1 receptor agonist, N^6-cyclohexyladenosine (CHA), may be related to their ability to stimulate glycogen synthesis and inhibit glycolysis and proton production during reperfusion following ischemia [1,2]. However, we have shown that in hearts stressed by transient ischemia, there is a loss of adenosine-induced cardioprotection [3] an effect consistent with the lower cardioprotective effectiveness of adenosine and adenosine mimetic agents in studies involving patients with coronary artery disease [4]. As this model of transient ischemia differs from ischemic preconditioning, in that the transient ischemia does not enhance the recovery of left ventricular function following severe ischemia [3], stressing by transient ischemia may provide a unique system to study the mechanisms underlying the loss of adenosine-induced cardioprotection observed in the clinical setting.

Alterations in glycogen metabolism and glucose utilization may contribute to the loss of adenosine-induced cardioprotection in stressed hearts. We reported previously that poor recovery of post-ischemic function is associated with an acceleration of glycolysis and proton production [3]. Adenosine-induced acceleration of glycolysis also occurs in stressed hearts during aerobic perfusion, suggesting that the increase in glycolysis, proton production and the potential for Na^+ and Ca^{2+} overload, is a cause, rather than a consequence, of depressed post-ischemic mechanical function. While the effects of adenosine on glycolysis have been extensively characterized, less well characterized are its effects on glycogen metabolism and the signaling pathways involved.

5'-AMP activated protein kinase (AMPK) is a multi-substrate enzyme involved in the control of cellular energy metabolism [5]. Upon activation in response to metabolic stresses, including hypoxia and ischemia, AMPK phosphorylates enzymes involved in the regulation of both fatty acid and glucose metabolism, thereby increasing ATP production [5]. Despite the importance of AMPK in regulating energy substrate metabolism, its role in regulating myocardial glycogen metabolism is not well understood. However in skeletal muscle, AMPK regulates glycogen content via mechanisms related to alterations in glucose uptake and the activity of glycogen synthase [6,7]. Furthermore, AMPK activity and glycogen content are inversely correlated in skeletal muscle [8], but whether such a reciprocal relationship exists in the heart has not been determined. Although adenosine does not stimulate glucose uptake in stressed hearts, it shifts the balance between the relative rates of glycogen synthesis and glycolysis, such that glucose taken up by the stressed myocardium is preferentially metabolized by glycolysis, and so contributes to an increase in proton production [9]. We have recently demonstrated that adenosine-induced acceleration of glycolysis and inhibition of glycogen synthesis in stressed hearts are accompanied by the activation of AMPK [9], thereby suggesting a relationship among glycogen synthesis, glycogen content and AMPK activity.

Recent reports suggest that p38 mitogen activated protein kinase (MAPK), another stress-responsive protein kinase, is activated downstream of AMPK and together, these kinases may regulate glucose uptake and subsequent myocardial glucose utilization [10,11,12,13]. Despite this established role of AMPK/p38 MAPK in glucose uptake, it is not known if AMPK and p38 MAPK form a functional signaling cascade in the regulation of glycogen metabolism and glycolysis. As the effects of adenosine on carbohydrate

metabolism are intimately linked to its cardioprotective properties, elucidation of involved biochemical mediators may provide novel protective strategies to enhance recovery of post-ischemic mechanical function.

This study investigated the involvement of AMPK and p38 MAPK in adenosine-induced alterations in glycogen and glucose metabolism in hearts stressed by transient ischemia using the selective p38 MAPK inhibitors, SB202190 and SB203580. Alterations in AMPK and p38 MAPK phosphorylation, as well as rates of glycogen and glucose metabolism were measured during aerobic perfusion of hearts previously stressed by transient ischemia where left ventricular (LV) work is stable, and cellular signaling events are not influenced by confounding factors such as energy substrate supply and energy demand.

4.2 Materials and Methods

4.2.1 Heart Perfusions

All animals received care according to the Canadian Council on Animal Care and the University of Alberta Health Sciences Animal Welfare Committee. Hearts from sodium pentobarbital-anesthetized male Sprague-Dawley rats (300 to 350 g) that had been fed *ad libitum*, were excised, their aortae were cannulated and a perfusion using Krebs-Henseleit solution (37°C, pH 7.4, gassed with a 95% O_2 - 5% CO_2 mixture) was initiated. Hearts were perfused in Langendorff mode for 10 min and thereafter switched to working (ejecting) mode as described previously [9]. The perfusate (recirculating volume of 100 mL) consisted of a modified Krebs-Henseleit solution containing 2.5 mM Ca^{2+}, 11 mM glucose, 1.2 mM palmitate pre-bound to 3% BSA (fatty acid free), and 100 mU/L insulin. Perfusions were performed at a constant workload (preload, 11.5 mm Hg;

afterload, 80 mm Hg) and heart rate (paced at 300 beats/min). Heart rate, systolic and diastolic aortic pressures (mm Hg), cardiac output and coronary flow were measured as described previously [9]. LV work (Joules) was calculated as cardiac output x LV developed pressure (systolic pressure - preload pressure) / 1000 x 0.133 and served as a continuous index of LV mechanical function.

4.2.2 Perfusion Protocol

Hearts were perfused under aerobic conditions for 15 min, then stressed by transient ischemia (two 10-min periods of global no-flow ischemia (unpaced) each followed by 5 min reperfusion). Hearts were then perfused aerobically for a further 35-min treatment period, either with vehicle (saline), SB202190 (10 μM), adenosine (500 μM), SB202190 (10 μM)/adenosine (500 μM), or SB203580 (10 μM/adenosine (500 μM). Thereafter, hearts were frozen for biochemical analyses (End-Treatment) (Fig. 4-1A).

4.2.3 Immunoblot Analysis of AMPK and p38 MAPK

Protein content of homogenates of frozen LV tissue, SDS-polyacrylamide gel electrophoresis, and immunoblotting with either rabbit anti-phospho-p38 MAPK (Thr180/Tyr182), rabbit anti-p38 MAPK (total), rabbit anti-phospho-AMPK (Thr172), or rabbit anti-AMPK-total (1:1000 dilution) in 5% BSA/1 x TBS were performed as previously described 9. Densitometric analyses of immunoblots (n=3 per experimental group) were performed using Quantity One (4.4.0) Software (Biorad Laboratories). Densitometric values in arbitrary density units (ADU) of the phosphorylated proteins are normalized to the total amount of the protein detected.

4.2.4 Measurement of Adenine Nucleotide Content

High-performance liquid chromatography (HPLC) was used to measure nucleotide and nucleoside content of neutralized perchloric acid extracts of frozen ventricular tissue as described previously [9].

4.2.5 Measurement of AMPK Activity

The activity of AMPK (nmol/mg protein/min) was measured in 6% polyethylene glycol (PEG) fractions extracted from 200 mg of frozen LV tissue by following the incorporation of [γ^{32}P] from [γ^{32}P]ATP into a ser-79 phosphorylation site-specific SAMS peptide (HMRSAMSGLHVKRR), as previously described [14,15,16].

4.2.6 Assay of Glycogen Content and Glucose Uptake

Glycogen, in 200 mg of powdered tissue, was converted to glucose by reacting with 4 M H_2SO_4. The amount of glucose (expressed as μmol glucose units/g dry wt) thus obtained was determined using a Sigma glucose analysis kit. The net rate of glycogen synthesis (μmol glucose/g dry wt/min) during the 35-min aerobic treatment period was calculated from the increase in [5-^{3}H]- and [^{14}C]glucosyl units in total glycogen in hearts frozen at End-Treatment relative to hearts frozen immediately following transient ischemia (Prior to Treatment) from [5-^{3}H]- and [^{14}C]glucose present in the perfusate. The rate of glucose uptake (μmol/g dry wt/min) during the treatment period was calculated as the sum of the net rate of glycogen synthesis and the rate of glycolysis in individual hearts [1,9]. The net rate of glycogen degradation was calculated as the difference between the unlabeled myocardial glycogen content in the Prior to Treatment and End-Treatment groups.

4.2.7 Measurement of Steady State Rates of Glycolysis and Glucose Oxidation

Glycolysis and glucose oxidation rates were measured directly from the simultaneous production of ^3H_2O and $^{14}CO_2$, respectively, from [5-^{3}H]glucose and [U-^{14}C]glucose, as described previously[1,2,9]. Perfusate was sampled at pre-determined time points (5, 14, 29, 44, 50, 60, 70, 80 min), and steady state rates, expressed as μmol [5-^{3}H]glucose or [U-^{14}C]glucose metabolized/g dry wt/min, were calculated for the aerobic treatment period.

4.2.8 Calculation of the Rate of Proton Production Arising from Exogenous Glucose Metabolism

When glucose is metabolized by glycolysis and completely oxidized the associated synthesis and hydrolysis of ATP results in a net proton production of zero. However, if the rate of glycolysis exceeds that of glucose oxidation, there is a net production of two protons per molecule of exogenous glucose that passes through glycolysis, which is not subsequently oxidized. Therefore the rate of proton production attributable to the hydrolysis of ATP arising from exogenous glucose metabolism can be calculated as 2 X (rate of glycolysis – rate of glucose oxidation).

4.2.9 Materials

D-[5-^{3}H]glucose and D-[U-^{14}C]glucose were purchased form Dupont Canada Inc., ON, Canada. SB202190 and SB203580 were purchased from Calbiochem, San Diego CA, USA. Adenosine was purchased from Research Biochemicals International, Natrick MA, USA. Anti-phospho-p38 MAPK (Thy180/Tyr182), anti-p38 MAPK (total), anti-phospho-AMPK (Thr172), and anti-AMPK (total) antibodies were obtained from Cell Signaling Technology, Beverly, MA, USA. All other chemicals were reagent grade.

4.2.10 Statistical Analysis

All values are presented as mean ± SE (n observations). The significance of the differences for multiple comparisons between treated and untreated groups was estimated by One-way Analysis of Variance (ANOVA). If significant, selected data sets were compared by Bonferroni's Multiple Comparison Test. Differences were considered significant when $P < 0.05$.

4.3 Results

4.3.1 Effects of p38 MAPK Inhibitors and Adenosine on LV Work and Coronary Flow

As reported previously for stressed hearts [3,9], LV work during the aerobic treatment period was not altered by adenosine (500 μM). LV work was also unaffected by the p38 MAPK inhibitor, SB202190 (10 μM), or by administration of SB202190 (10 μM) and adenosine (500 μM), or SB203580 (10 μM) and adenosine (500 μM) (Fig. 4-1B). Furthermore, coronary flow was not affected by any of these treatments Table 4-1).

4.3.2 Effects of p38 MAPK Inhibitors on the Phosphorylation of AMPK and p38 MAPK

As described previously, transient ischemia *per se* does not alter the phosphorylation of AMPK compared to hearts perfused in the absence of transient ischemia [9]. Thus, the possible involvement of p38 MAPK was assessed during the aerobic treatment period, in the presence or absence of adenosine. Perfusion of hearts with SB202190 alone did not affect the phosphorylation of AMPK compared with hearts treated with vehicle. Consistent with our previous findings, adenosine caused a marked increase in the phosphorylation of AMPK. The adenosine-induced increase in AMPK

phosphorylation was abrogated by co-treatment with either SB202190 or SB203580 (Fig. 4-2A). The changes in AMPK phosphorylation were consistent with changes in AMPK activity (Fig. 4-2B). A similar pattern of changes in phosphorylation was observed for p38 MAPK. SB202190 alone did not affect p38 MAPK phosphorylation compared with hearts treated with vehicle, while adenosine increased p38 MAPK phosphorylation. The adenosine-induced increase in p38 MAPK phosphorylation was eliminated by either SB202190 or SB203580 (Fig. 4-2B).

4.3.3 Effects of p38 MAPK Inhibitors and Adenosine on Nucleoside and Nucleotide Content

The content of adenosine and adenine nucleotides in LV tissue was determined to characterize potential mechanisms responsible for alterations in p38 MAPK and AMPK phosphorylation (Table 4-2). SB202190 alone did not alter adenosine content compared with vehicle-treated hearts. As expected, exogenous adenosine significantly increased adenosine content, which was not altered by either SB202190 or SB203580. None of the treatments significantly altered either ATP or AMP content, or the ATP/AMP ratio, suggesting that the mechanisms underlying alterations AMPK and p38 MAPK phosphorylation occurred independently of changes in nucleotide content (Table 4-2).

4.3.4 Effects of p38 MAPK Inhibitors on AMPK Activity

To assess any direct effects of SB202190 and SB203580 on AMPK activity, graded concentrations (0 to 100 μM) of each p38 MAPK inhibitor were included in PEG fractions isolated from hearts with an elevated AMPK activity. Neither of the compounds had any direct effect on AMPK activity (Fig. 4-3A and Fig. 4-3B).

4.3.5 Effects of p38 MAPK Inhibitors on Glucose Uptake and Glycogen Metabolism

A previous study has described the effects of adenosine on the rates of glucose uptake, glycogen synthesis, glycogen degradation, and glycogen content in hearts stressed by transient ischemia [9]. A portion of those data (vehicle-treated and adenosine-treated hearts) are restated here to facilitate comparison with effects of the p38 MAPK inhibitors. The rate of glucose uptake during the aerobic treatment period was similar to that reported previously for stressed hearts and was not affected by either adenosine, SB202190 or adenosine in combination with the p38 MAPK inhibitors (Fig. 4-4A). While SB202190 did not affect the rate of glycogen synthesis compared with vehicle-treated hearts, SB202910 and SB203580 each prevented the adenosine-induced suppression of glycogen synthesis (Fig. 4-4B). The rate of glycogen degradation was not altered by any of the treatments (Fig. 4-4C). Consequently, glycogen content was significantly greater in hearts treated with a combination of either SB202190/adenosine or SB203580/adenosine compared with hearts treated with adenosine alone (Fig. 4-4D). As a result of the changes in glycogen metabolism, there was a significant inverse correlation between the rate of glycogen synthesis and AMPK activity (r^2=0.94, P<0.05) (Fig. 4-5A) and AMPK activity and glycogen content (r^2=0.93, P<0.05) (Fig. 4-5B).

4.3.6 Effects of p38 MAPK Inhibitors on Glucose Metabolism

While SB20190 alone did not affect the rate of glycolysis, the marked adenosine-induced acceleration of glycolysis, that was described previously [9], was abolished by either SB202190 or SB203580 (Fig. 4-6A). The rate of glucose oxidation was not altered by any of the treatment combinations (Fig. 4-6B). The rate of proton production, which

is an index of uncoupling of glycolysis and glucose oxidation, was increased by adenosine as reported previously [3,9] and was not affected by SB20190 alone. However, SB202190 or SB203580 each prevented the adenosine-induced stimulation of the rate of proton production (Fig 4-6C).

4.4 Discussion

4.4.1 Summary of Major Findings

LV work is stable during aerobic perfusion of hearts stressed by transient ischemia, but there is a loss of adenosine-induced cardioprotection following a subsequent period of severe ischemia, possibly due to changes in adenosine-mediated alterations in myocardial glucose metabolism. This study investigated the roles of AMPK and p38 MAPK in the regulation of glycogen metabolism and glucose utilization in stressed hearts, a system where adenosine activates AMPK and accelerates glycolysis. While the p38 MAPK inhibitor, SB202190, did not affect the phosphorylation of AMPK or p38 MAPK in the absence of adenosine, SB202190 and SB203580 each abolished the adenosine-induced increase in both AMPK and p38 MAPK phosphorylation. The p38 MAPK inhibitors also inhibited the adenosine-induced acceleration of glycolysis and the adenosine-induced suppression of glycogen synthesis. In contrast to previous reports that describe p38 MAPK to be downstream of AMPK in the regulation of glucose metabolism [10,11,13], we provide evidence using selective p38 MAPK inhibitors that p38 MAPK is upstream of AMPK and that its activation stimulates AMPK phosphorylation and activity. The ability of the p38 MAPK inhibitors to prevent AMPK activation by adenosine as well as its consequences on glycolysis and glycogen synthesis indicates that

p38 MAPK is upstream of AMPK in the pathway that alters myocardial glucose utilization in response to adenosine (Fig. 4-7).

4.4.2 p38 MAPK Inhibitors Prevent the Adenosine-Induced Activation of AMPK and p38 MAPK

The ability to measure glycogen metabolism and glucose utilization during aerobic conditions and stable LV mechanical function and coronary flow allowed the characterization of AMPK and p38 MAPK signaling events involved in their regulation independent of confounding factors such as differences in energy supply, O_2 availability or energy demand. Inhibitors of p38 MAPK prevented adenosine-induced increase in both AMPK and p38 MAPK phosphorylation, but their mechanism has not been clearly defined. Although a previous report demonstrates that both SB202190 and SB203580 inhibit adenosine uptake in cultured human erythroleukemia (K562) cells [17], and thus may prevent adenosine-induced alterations in the ATP/AMP ratio, they had no significant effect on either adenosine uptake or adenine nucleotide content in this study. As the reported ventricular adenosine content in this study represents total tissue adenosine, it takes into account both intracellular and extracellular adenosine content. However, as the biological half-life of adenosine is extremely short, the reported adenosine content likely represents adenosine in the extracellular space. Interestingly α-adrenoceptors are involved in activating both AMPK and p38 MAPK [11,18], and the α-adrenoceptor antagonist, phentolamine, prevents the adenosine-induced increase in AMPK phosphorylation in stressed hearts [9]. Thus, α-adrenoceptors may participate in the alterations in both AMPK and p38 MAPK phosphorylation in response to adenosine and the p38 MAPK inhibitors, and the subsequent regulation of glucose utilization (Fig. 4-7).

4.4.3 p38 MAPK is Upstream of AMPK in Stressed Hearts Treated With Adenosine

Similar to previous reports, our results indicate that AMPK and p38 MAPK form a common signaling cascade and participate in the regulation of glucose metabolism [10,11,13]. Interestingly previous reports have implicated transforming growth factor-β-activated protein kinase 1-binding protein 1 (TAB1) as a MAPK kinase (MEK) 3 independent mechanism of p38 MAPK activation [21]. This mechanism appears to require AMPK to facilitate the recruitment of p38 MAPK to TAB1 macromolecular complexes prior to subsequent p38 MAPK autophosphorylation in the isolated perfused mouse heart. However, TAB1 may not play a prominent role in the activation of p38 MAPK in the rat heart, as endogenous TAB1 protein is not detectable by western immunoblotting techniques in isolated neonatal rat cardiac myocytes [22]. Furthermore, our results in isolated rat hearts stressed by transient ischemia and subsequent treatment with adenosine suggest that AMPK is downstream of p38 MAPK. This conclusion is based on the observation that SB202190 and SB203580 inhibit adenosine-induced activation of both p38 MAPK and AMPK. It is unlikely that the inhibition of AMPK was mediated directly by these compounds as they compete with ATP for binding to the ATP-binding pocket of p38 MAPK that contains amino acid residues distinct from those found in the ATP-binding pocket of AMPK [23,24]. Moreover, the selectivity of SB202190 and SB203580 versus a variety of other protein kinases including AMPK has been validated previously [25]. We have further confirmed that these p38 MAPK inhibitors have no direct effect on AMPK activity in our preparations, as AMPK retains 94-96% of its activity *in vitro* in the presence of SB202190 (10 μM) or SB203580 (10 μM). Thus, our data strongly support

the observation that p38 MAPK is upstream of AMPK in the signaling cascade linking adenosine with changes in myocardial glycogen metabolism and glycolysis (Fig. 4-7). Although this study did not assess the activity of LKB1, a recently identified AMPK kinase (AMPKK), which appears not to be responsive to metabolic stresses including hypoxia or ischemia [19,20], or the activity of other alternate, as yet to be identified AMPKKs, our results strongly support the observation that p38 MAPK is upstream of AMPK in the signaling cascade linking adenosine with changes in myocardial glycogen metabolism and glycolysis.

4.4.4 p38 MAPK and AMPK do not Affect Myocardial Glucose Uptake

Myocardial glucose uptake is stimulated by metabolic stresses, including hypoxia and ischemia, by insulin-independent mechanisms [12]. AMPK and p38 MAPK also regulate glucose uptake in response to metabolic stresses [12,26,27] possibly by distinct mechanisms. While AMPK stimulates glucose uptake by increasing the translocation of GLUT4 transporters to the cell surface [26,27,28,29], p38 MAPK may stimulate glucose uptake by increasing the intrinsic activity of GLUT4 transporters already at the cell surface [30,31,32]. Despite these potential synergistic effects of AMPK (GLUT4 translocation) and p38 MAPK (GLUT4 activation), differences in glucose uptake were not observed in stressed hearts despite marked differences in the extent of AMPK and p38 MAPK phosphorylation. The lack of effect of SB202190 and SB203580 on glucose uptake despite their ability to abrogate the adenosine-induced increase in both AMPK and p38 MAPK suggests that these two kinases do not significantly regulate glucose uptake in the fatty acid perfused working rat heart during aerobic conditions.

Therefore, our data contrast with reports suggesting a requirement for AMPK activation in ischemia-induced stimulation of myocardial glucose uptake and for p38 MAPK phosphorylation in the acceleration of glucose uptake in adipocytes, myotubes, isolated adult cardiomyocytes, and the isolated perfused mouse heart [11,33,34]. It should be noted, that in the current study, AMPK and p38 MAPK activation was achieved during non-ischemic conditions where the supply of glucose is not rate-limiting. Moreover, isolated cardiomyocytes have minimal energy requirements; rates of oxidative metabolism are 50 to 100-fold less than in isolated working rat hearts, and therefore do not represent a normal energy demand [35]. Thus, as hearts in this study had a near physiological energy demand, glucose utilization is high, but not maximal [36]. Under these more physiological conditions of appropriate energy substrate supply and energy demand glucose uptake appears insensitive to changes in p38 MAPK or AMPK activity. This observation is further supported by a previous study which demonstrates that the activation of cardiac AMPK *in vivo* is not sufficient to increase glucose clearance [37].

4.4.5 p38 MAPK and AMPK Regulate Glycogen Metabolism in Stressed Hearts Treated with Adenosine

Although glucose uptake was similar among experimental groups, there were important differences in glucose utilization with regards to glycogen synthesis, total glycogen content, as well as glycolysis and proton production. Although the p38 MAPK inhibitor, SB202190, alone did not affect the rate of glycogen synthesis, SB202190 or SB203580 each prevented the adenosine-induced inhibition of glycogen synthesis. The restoration of normal rates of glycogen synthesis by the p38 MAPK inhibitors allowed glycogen to accumulate normally during the aerobic treatment period. Thus under these

conditions, we have shown that AMPK activation inhibits glycogen synthesis and that inhibition of p38 MAPK prevents AMPK activation, restores glycogen synthesis and allows glycogen to accumulate to normal levels. To our knowledge, this is the first report to demonstrate a significant inverse correlation between myocardial glycogen content and AMPK and p38 MAPK activities, and supports a study by Wojtaszewski et al. that demonstrates an inverse relationship between the activities of glycogen synthase and AMPK, as well as glycogen content and AMPK activity in rat skeletal muscle [8]. Thus glycogen content becomes an important consideration in investigations of cardiac AMPK activity.

The prevention of adenosine-induced inhibition of glycogen accumulation by SB202190 or SB203580 resembles the profile of glucose utilization observed in normal hearts with low AMPK activity, where the balance in the fate of glucose between glycogen synthesis and glycolysis favors glycogen synthesis. Glycogen accumulation and the preferential oxidation of glucose liberated from glycogen may provide an energetic advantage and it also lessens the potential for glycolysis and proton production [38,39]. Glycogen synthesis is an important target in CHA-induced cardioprotection [1], but whether the ability of p38 MAPK inhibitors to relieve the adenosine-induced suppression of glycogen synthesis in stressed hearts translates into enhanced cardioprotection following severe ischemia remains to be determined.

4.4.6 Conclusions

In conclusion, in hearts stressed by transient ischemia, adenosine activates both AMPK and p38 MAPK. These effects are inhibited by either of the p38 MAPK inhibitors, SB202190 or SB203580, and are unrelated to changes in nucleotide content.

As the p38 MAPK inhibitors lack any direct effect on AMPK activity, the results suggest that p38 MAPK is upstream of AMPK in this system. In contrast to previous reports, alterations in both AMPK and p38 MAPK phosphorylation do not affect glucose uptake; rather, the consequences of their activation are manifest as an alteration in the partitioning of glucose between glycogen synthesis and glycolysis. The inverse correlation between AMPK activity and myocardial glycogen synthesis and glycogen content was associated with an attenuation of the rates of glycolysis and proton production. These results suggest that the cardioprotective effectiveness of adenosine that is lost in hearts stressed by transient ischemia may be due to activation of AMPK [3]. As there is currently a lack of selective AMPK inhibitors, inhibitors of p38 MAPK may be useful to restore adenosine-induced cardioprotection following further ischemic challenge via the downstream inhibition of AMPK that elicits salutary alterations in glucose metabolism.

Figure 4-1. Experimental protocol for heart perfusions. A, Hearts were perfused aerobically for 15 min prior to stressing by transient ischemia (two 10-min periods of global no-flow ischemia (shaded bars) each followed by 5 min reperfusion). Stressed hearts were then either frozen for biochemical analyses prior to treatment, or were assigned randomly to groups treated with vehicle (saline, n=8), SB202190 (10 μM, n=6), adenosine (500 μM, n=7), SB202190 (10 μM)/adenosine (500 μM, n=8), or SB203680 (10 μM)/adenosine (500 μM, n=10) and perfused aerobically for a further 35-min aerobic treatment period. Hearts were then frozen for biochemical measurements (End-Treatment, ↓). B, LV work of hearts perfused in the absence or presence of adenosine, SB202190, and SB203580 (indicated by – and + , respectively). Values represent means ± SE.

Figure 4-1

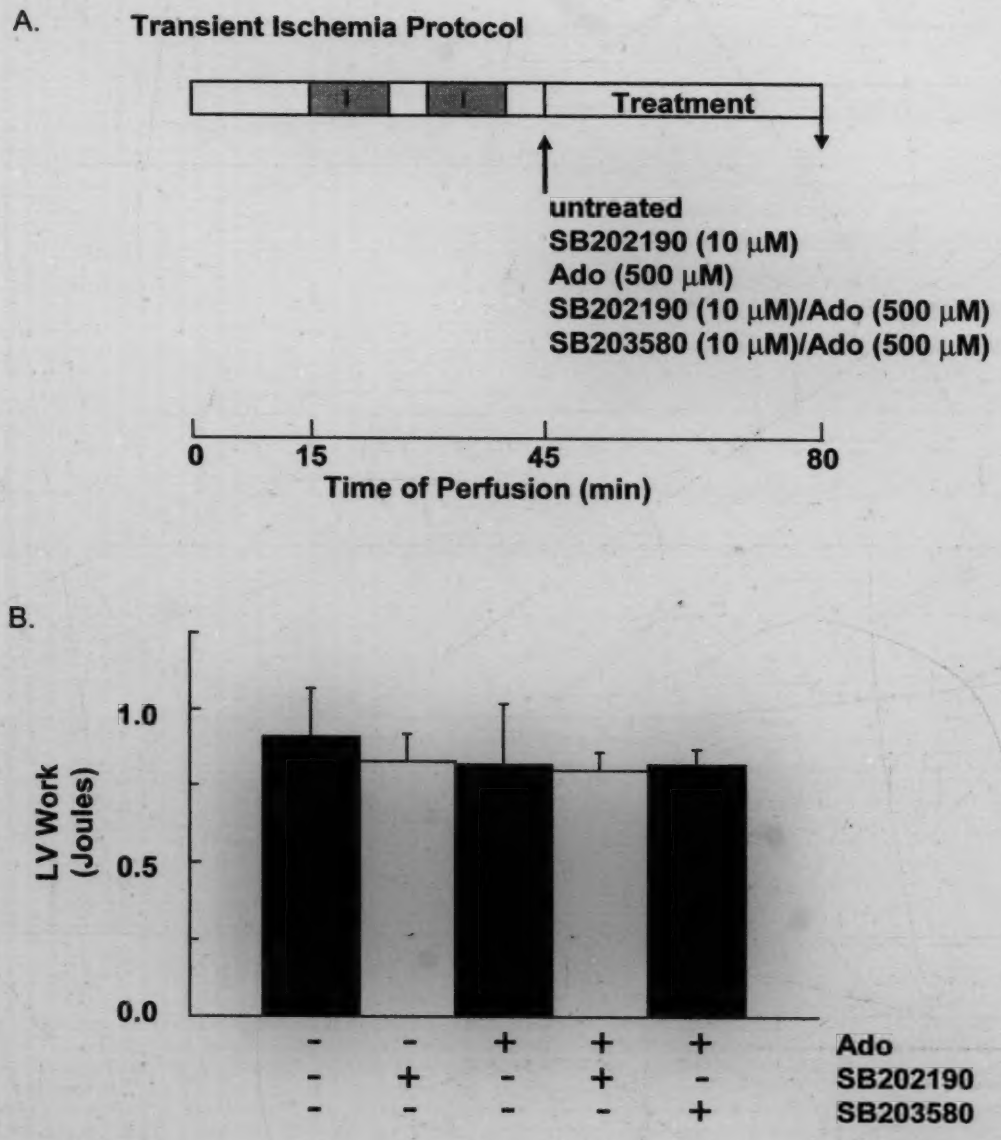
A.
Transient Ischemia Protocol
I
I
Treatment
untreated
SB202190 (10 μM)
Ado (500 μM)
SB202190 (10 μM)/Ado (500 μM)
SB203580 (10 μM)/Ado (500 μM)
0
15
45
80
Time of Perfusion (min)
B.
LV Work (Joules)
1.0
0.5
0.0
- - + + + Ado
- + - + - SB202190
- - - - + SB203580

Figure 4-2. AMPK and p38 MAPK phosphorylation in vehicle-treated stressed hearts. Immunoblots and their densitometric analysis (ADU) were assessed in hearts frozen at End-Treatment as described under "Materials and Methods." A, *Top panel,* representative immunoblots of phosphorylated AMPK and total AMPK from ventricular homogenates. *Bottom panel,* densitometric analysis of immunoblots (n=3 per group). B, AMPK activity (n=3 per group) in hearts frozen at End-Treatment as described under "Materials and Methods." C, *Top panel,* representative immunoblots of phosphorylated p38 MAPK and total p38 MAPK from ventricular homogenates. *Bottom panel,* densitometric analysis of immunoblots (n=3 per group). The absence or presence of adenosine, SB202190, and SB203580 is indicated by – and +, respectively. Values represent means ± SE. * indicates a significant difference from vehicle-treated hearts; † indicates a significant difference from adenosine-treated hearts.

Figure 4-2

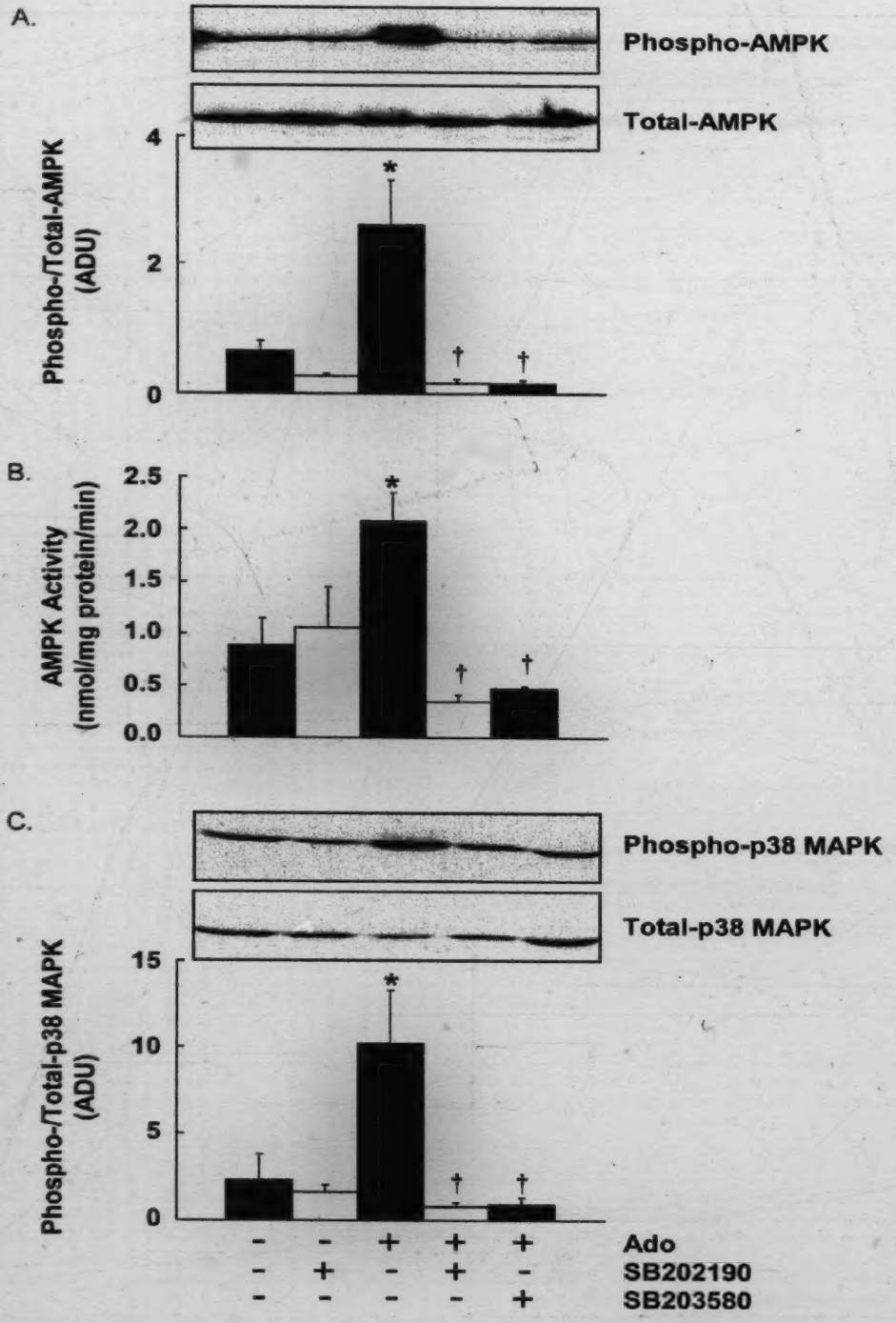
A.
Phospho-AMPK
Total-AMPK
Phospho-/Total-AMPK (ADU)
4
2
0
*
†
†
B.
AMPK Activity (nmol/mg protein/min)
2.5
2.0
1.5
1.0
0.5
0.0
*
†
†
C.
Phospho-p38 MAPK
Total-p38 MAPK
Phospho-/Total-p38 MAPK (ADU)
15
10
5
0
*
†
†
- - + + + Ado
- + - + - SB202190
- - - - + SB203580

Figure 4-3. Direct effects of p38 MAPK inhibitors on AMPK activity. Lack of effect of SB202190 (A, 0 to 100 μM, n=3) and SB203580 (B, 0 to 100 μM, n=3) on the activity of AMPK in ventricular homogenates (expressed as a % of activity in absence of inhibitors). Values represent means ± SE.

Figure 4-3

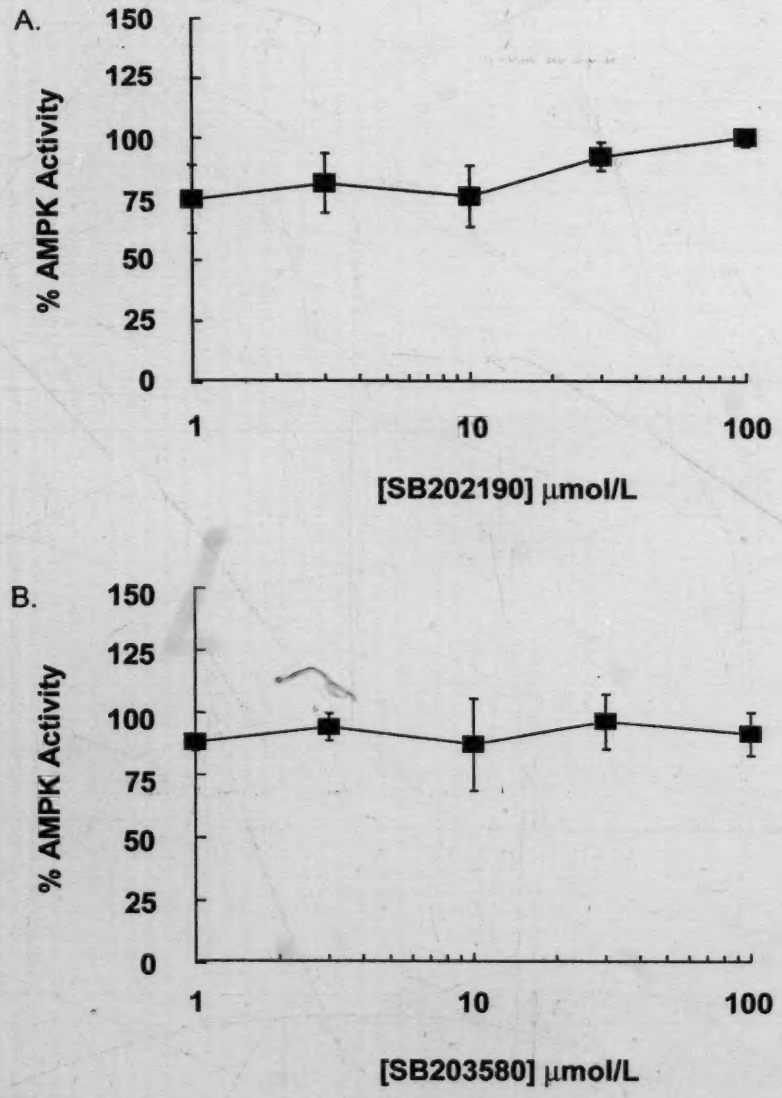
A.
% AMPK Activity
150
125
100
75
50
25
0
1
10
100
[SB202190] μmol/L
B.
% AMPK Activity
150
125
100
75
50
25
0
1
10
100
[SB203580] μmol/L

Figure 4-4. Glucose uptake and glycogen metabolism in stressed hearts. Glucose uptake (A), glycogen synthesis (B), glycogen degradation (C) and glycogen content (D) were assessed as described under "Materials and Methods" for hearts treated with vehicle (n=6), SB202190 (10 μM, n=6), adenosine (500 μM, n=6), SB202190 (10 μM)/adenosine (500 μM, n=6), or SB203580 (10 μM)/adenosine (500 μM, n=5). The absence or presence of adenosine, SB202190, and SB203580 is indicated by – and + , respectively. Values represent means ± SE. * indicates a significant difference from vehicle-treated hearts; † indicates a significant difference from adenosine-treated hearts.

Figure 4-4

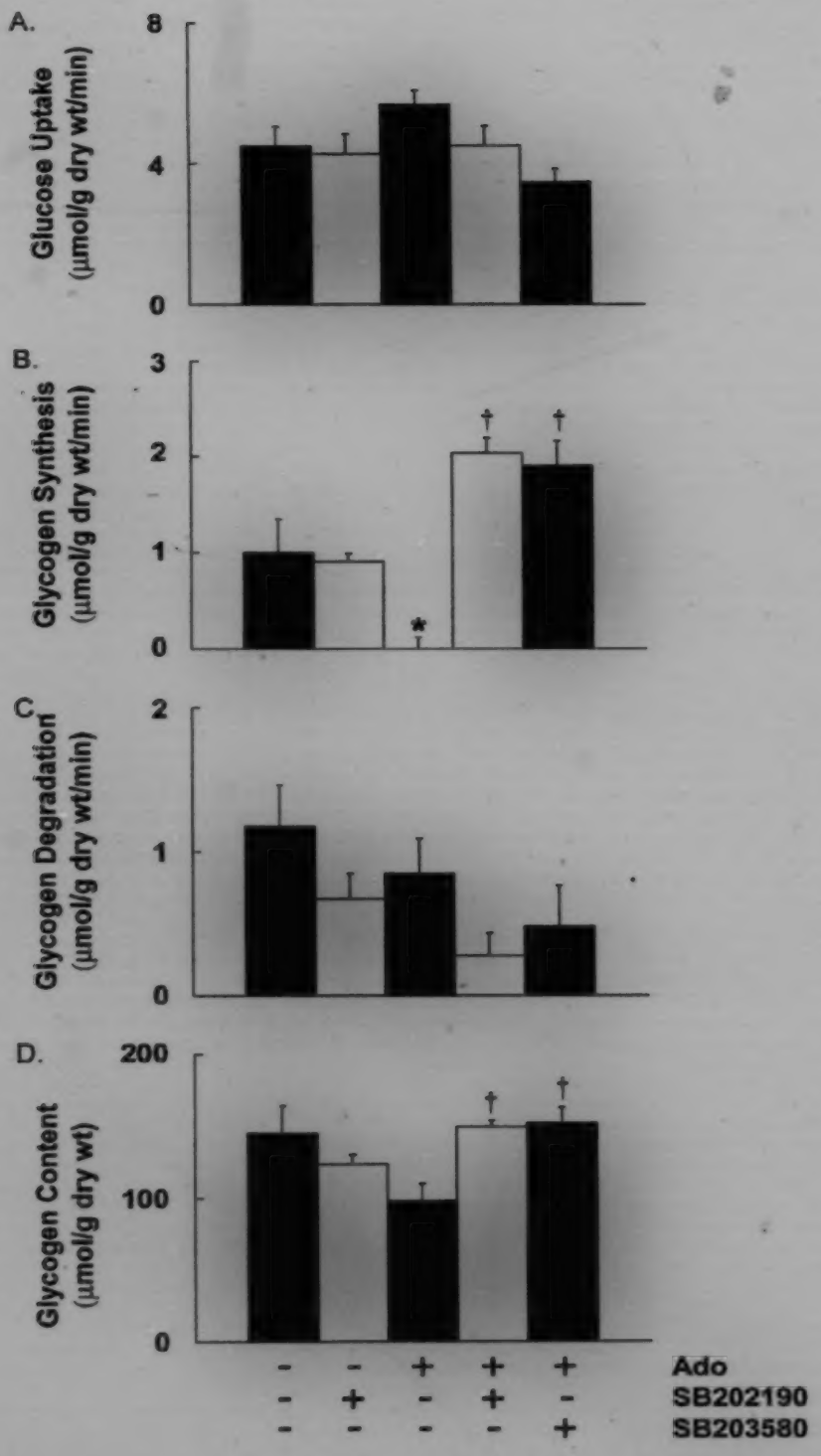
A.
Glucose Uptake
(μmol/g dry wt/min)
8
4
0
B.
Glycogen Synthesis
(μmol/g dry wt/min)
3
2
1
0
*
†
†
C
Glycogen Degradation
(μmol/g dry wt/min)
2
1
0
D.
Glycogen Content
(μmol/g dry wt)
200
100
0
†
†
- - + + +
Ado
- + - + -
SB202190
- - - - +
SB203580

Figure 4-5. AMPK activity and indices of glycogen metabolism in stressed hearts. Correlation between AMPK activity in ventricular homogenates of hearts frozen at End-Treatment and the rate of glycogen synthesis calculated during the treatment period (A, n=3-6 per group and glycogen content measured at the end of the treatment period (B, n=3-6 per group). Hearts treated with: vehicle (○), SB202190 (□), adenosine (●), SB202190/adenosine (■), or SB203580/adenosine (▲). Values represent means ± SE.

Figure 4-5

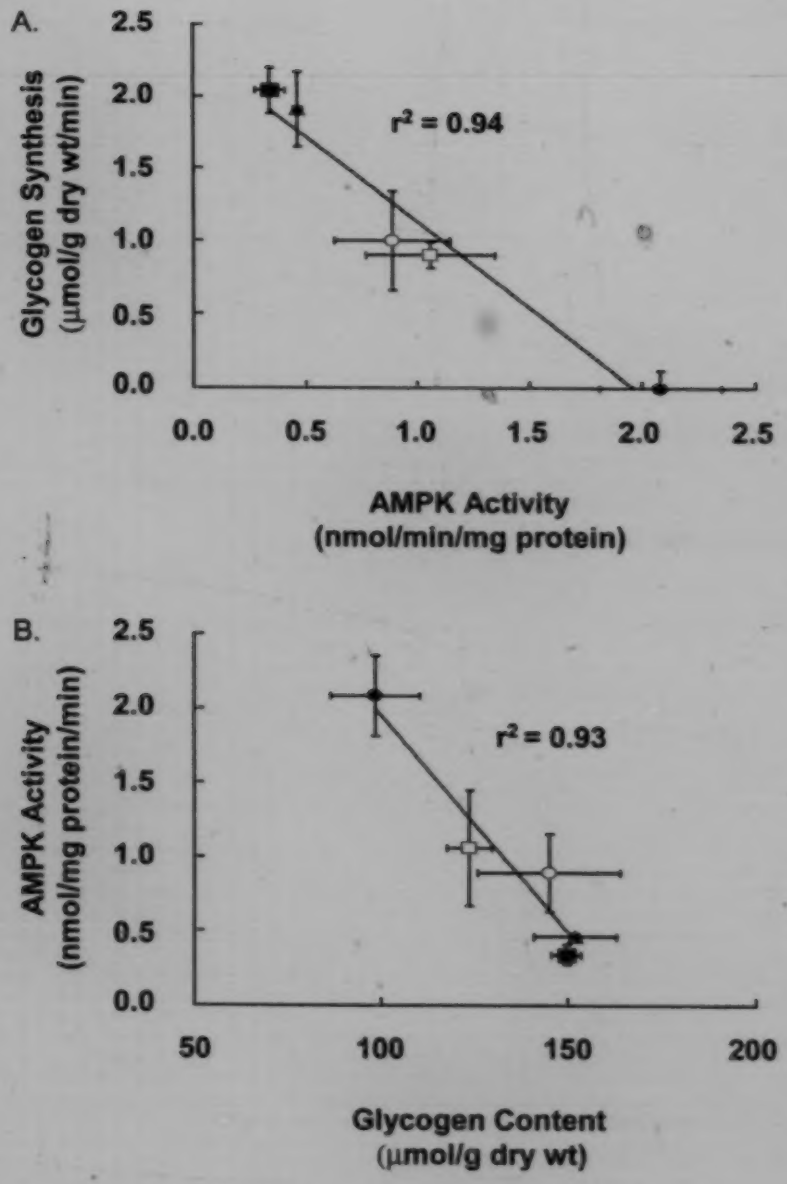
A.
Glycogen Synthesis
(μmol/g dry wt/min)
r² = 0.94
AMPK Activity
(nmol/min/mg protein)
B.
AMPK Activity
(nmol/mg protein/min)
r² = 0.93
Glycogen Content
(μmol/g dry wt)

Figure 4-6. Rates of glucose metabolism and proton production in stressed hearts. Rates (μmol glucose/g dry wt/min) of glycolysis (A), glucose oxidation (B) and proton production from glucose metabolism (C) were assessed as described under "Materials and Methods" for hearts treated with vehicle (n=10), SB202190 (10 μM, n=6), adenosine (500 μM, n=7), SB202190 (10 μM)/adenosine (500 μM, n=8), or SB203580 (10 μM)/adenosine (500 μM, n=10). Values represent means ± SE. * indicates a significant difference from vehicle-treated hearts; † indicates a significant difference from adenosine-treated hearts.

Figure 4-6

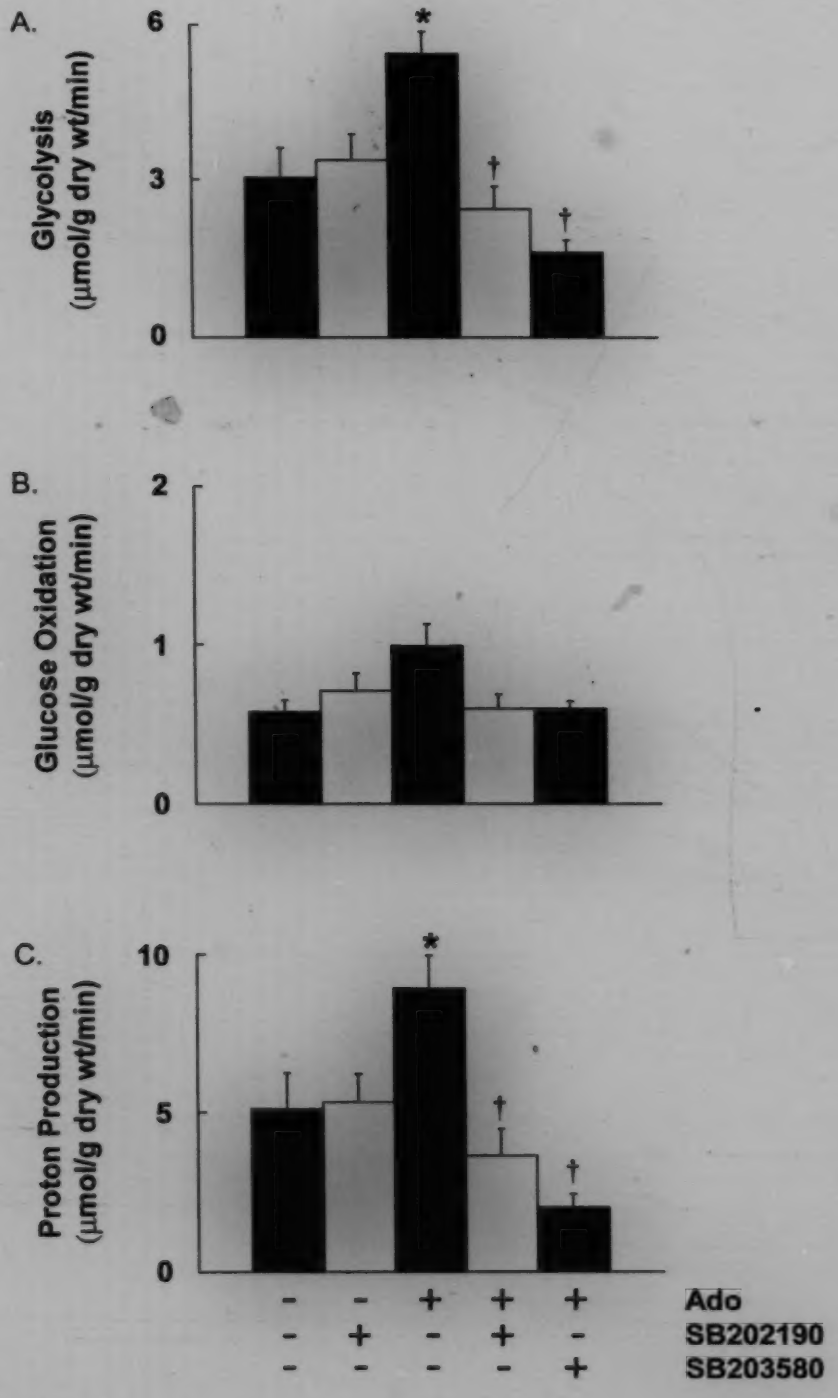
A.
Glycolysis
(μmol/g dry wt/min)
6
3
0
*
†
†
B.
Glucose Oxidation
(μmol/g dry wt/min)
2
1
0
C.
Proton Production
(μmol/g dry wt/min)
10
5
0
*
†
†
- - + + +
- + - + -
- - - - +
Ado
SB202190
SB203580

Figure 4-7. Schematic illustration of the effects of adenosine on glucose utilization in stressed hearts. Adenosine, by a nucleotide–independent mechanism that may involve modification of α-adrenoceptor activation or transduction (❶), stimulates p38 MAPK (❷) by a mechanism inhibitable by SB202190 or SB203580 (❸). Activation of p38 MAPK stimulates AMPK (❹). Activation of these kinases does not affect glucose uptake, but alters the balance of glucose utilization between glycogen synthesis and glycolysis, such that glycogen synthesis is suppressed and glycolysis is accelerated (❺). As the rate of glucose oxidation remains unchanged, there is an uncoupling of rates of glycolysis and glucose oxidation, such that protons (H^+) derived from the hydrolysis of glycolytically-derived ATP are no longer consumed by the tricarboxylic acid (TCA) cycle (❻). The resulting accumulation of protons leads to intracellular acidosis and an acceleration of the exchange of intracellular H^+ for extracellular Na^+ via the Na^+/H^+ exchanger (NHE) (❼). Intracellular Na^+ accumulation in turn leads to Ca^2 accumulation via activation of the Na^+/Ca^{2+} exchanger (NCX) (❽). Inhibition of p38 MAPK does not change adenine nucleotide contents, but prevents the adenosine-induced activation of p38 MAPK. AMPK is no longer activated and the adenosine-induced suppression of glycogen synthesis and acceleration of glycolysis are prevented. Thus, p38 MAPK inhibitors have the potential to restore adenosine-induced cardioprotection in stressed hearts.

Figure 4-7

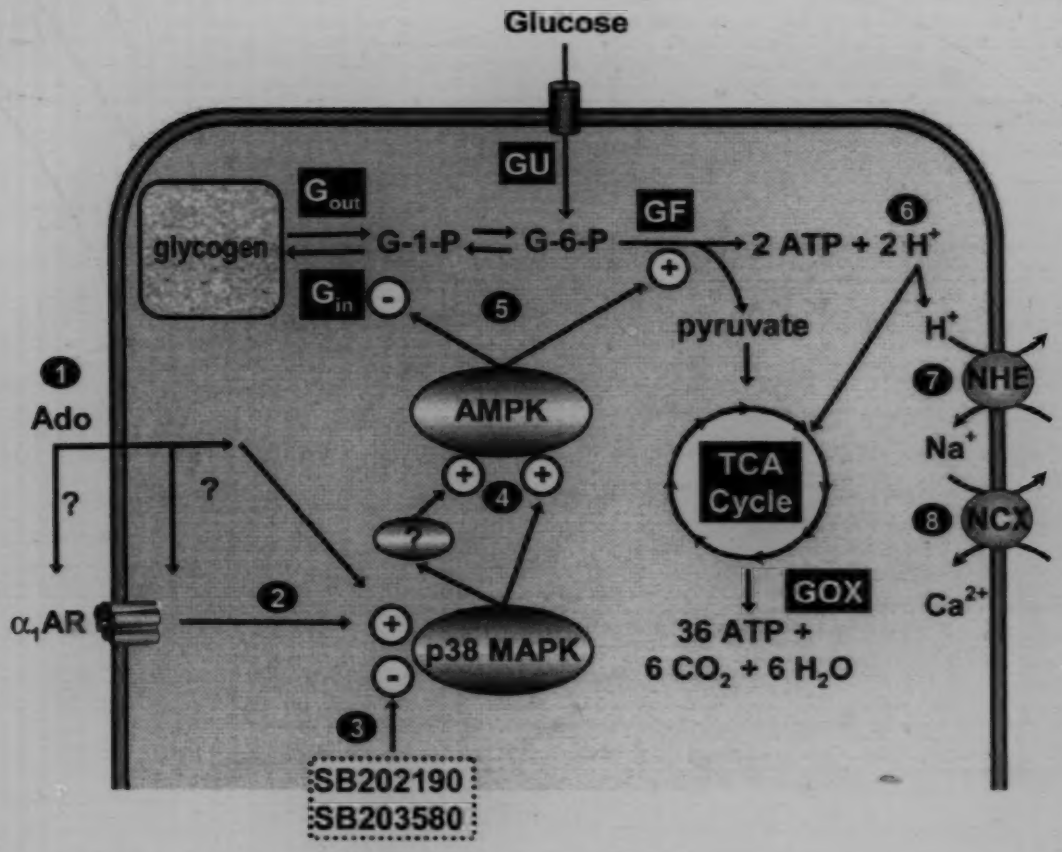
Glucose
GU
G_{out}
glycogen
G-1-P
G-6-P
GF
2 ATP + 2 H^+
G_{in}
5
pyruvate
H^+
1
Ado
AMPK
TCA Cycle
7
NHE
Na^+
?
?
4
?
8
NCX
Ca^{2+}
α_1AR
2
GOX
p38 MAPK
36 ATP +
6 CO_2 + 6 H_2O
3
SB202190
SB203580

Table 4-1. Coronary flow in stressed hearts.

Experimental Group	Coronary Flow (ml/min)
Vehicle	26.4±3.0 (8)
SB202190	21.4±0.9 (6)
Ado	27.2±1.9 (7)
SB202190/Ado	27.5±1.0 (8)
SB203580/Ado	24.9±1.4 (10)

Coroary flow (mL/min) was measured in stressed hearts treated with vehicle, SB202190, Ado, SB202190/Ado, and SB203580/Ado. Values represent means ± SE.

Table 4-2. Adenosine and adenine nucleotide content in stressed hearts.

Treatment	Adenosine	ATP	AMP	ATP/AMP
Vehicle	0.18 ± 0.04 (6)	13 .69 ± 2.1	4.61 ± 0.3 (6)	3.03 ± 0.5 (6)
SB202190	0.15 ± 0.02 (6)	18.55 ± 2.1 (6)	4.00 ± 0.4 (6)	4.94 ± 0.7 (6)
Ado	2.97 ± 0.4 (6)*	19.47 ± 2.2 (6)	8.57 ± 2.5 (6)	3.07 ± 0.7 (6)
SB202190/Ado	2.24 ± 0.1 (6)*	16.46 ± 0.9 (6)	4.35 ± 1.3 (6)	3.78 ± 0.5 (6)
SB203580/Ado	3.09 ± 0.3 (6)*	19.52 ± 2.5 (6)	6.45 ± 1.3 (4)	3.44 ± 0.4 (4)

Adenosine (Ado) and adenine nucleotide contents (μmol/g dry wt) in perchloric acid extracts from ventricular tissue from stressed hearts frozen at the end of the aerobic treatment period. * indicates a significant difference from vehicle-treated hearts.

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Chapter 5

Inhibition of p38 Mitogen Activated Protein Kinase and 5'-AMP Activated Protein Kinase Restores Adenosine-Induced Cardioprotection in Stressed Hearts by Altering Glucose Utilization

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5.1 Introduction

Adenosine, an endogenous purine nucleoside, has been shown to be an effective pharmacological approach to limit cardiac injury during reperfusion following ischemia. Ischemia itself causes numerous disturbances, particularly an imbalance between energy substrate supply and energy substrate demand. The ability of adenosine to improve the recovery of post-ischemic myocardial function may in part be related to its ability to restore the balance between energy substrate supply (coronary vasodilation) and energy demand (negative inotropism and chronotropism) [1,2], as well as its effects on myocardial carbohydrate metabolism. Previous reports demonstrate that adenosine-induced cardioprotection, manifest as an improved recovery of post-ischemic mechanical function is accompanied by an inhibition of glycolysis and calculated proton (H^+) production [3,4,5], which may serve to attenuate intracellular acidosis. This in turn reduces the potential for Na^+ and Ca^{2+} overload that contribute to depressed mechanical function during reperfusion [6,7].

Interestingly, if hearts are stressed by transient, antecedent ischemia (two cycles of 10-min ischemia and 5-min reperfusion) prior to the onset of severe global ischemia, the effects of adenosine on glucose metabolism are reversed, such that glycolysis is stimulated. As the rate of glucose oxidation remains unchanged, coupling between glycolysis and glucose oxidation is reduced and this leads to an acceleration of the rate of myocardial H^+ production [8]. As a consequence, the recovery of LV work is impaired by adenosine in hearts stressed by transient ischemia [8]. Thus stressing with transient ischemia may produce a model similar to the clinical scenario where adenosine mimetic compounds have limited cardioprotective effectiveness [9].

We have recently described that the adenosine-induced acceleration of glycolysis and H^+ production during aerobic perfusion of stressed hearts is accompanied by a suppression of the rate of glycogen synthesis and an activation of the 5'-AMP activated protein kinase (AMPK) [10]. Furthermore, we have demonstrated that inhibition of p38 mitogen activated protein kinase (MAPK) inhibits the activation of AMPK, attenuates the rates of glycolysis and H^+ production, as well as relieving the suppression of glycogen synthesis [11]. Whether this mechanism persists in stressed hearts during reperfusion following severe ischemia and translates into cardioprotection has yet to be determined.

p38 MAPK and AMPK are stress-responsive protein kinases involved in ischemia reperfusion (IR) injury and in the regulation of glucose utilization. In addition, these two kinases form a functional signaling cascade [12,13]. The roles of both p38 MAPK and AMPK in myocardial IR injury are complex. With regards to p38 MAPK and the recovery of post-ischemic myocardial function there is controversy, with studies suggesting that p38 MAPK activation is beneficial [14,15], while other studies suggest that its activation is detrimental, and that its inhibition during reperfusion is cardioprotective [16,17,18,19]. Similarly, the role of AMPK in the evolution of IR injury is not clearly defined. Previous studies suggest that the activation of AMPK is cardioprotective via inhibition of apoptosis, and via increased glucose uptake and glycolysis [20,21]. However, increased rates of glycolysis during reperfusion coupled with the rapid recovery of fatty acid oxidation at the expense of glucose oxidation accelerates the calculated rate of myocardial H^+ production and so contributes to depressed contractile function and cardiac efficiency during reperfusion [22,23].

In this study we investigated whether the inhibition of p38 MAPK can inhibit AMPK as well as glycolysis and H^+ production in stressed hearts during reperfusion following severe ischemia. In accordance with our findings in stressed hearts during aerobic perfusion, we hypothesized that inhibition of p38 MAPK will inhibit AMPK, attenuate glycolysis and H^+ production, and thus restore the cardioprotective effectiveness of adenosine in stressed hearts during reperfusion following severe ischemia.

5.2 Materials and Methods

5.2.1 Heart Perfusions

Hearts from sodium pentobarbital-anesthetized male Sprague-Dawley rats (300 to 350 g) that had been fed *ad libitum*, were excised, their aortae were cannulated and a perfusion using Krebs-Henseleit solution (37°C, pH 7.4, gassed with a 95% O_2 - 5% CO_2 mixture) was initiated. Hearts were perfused in the Langendorff mode for 10 min and thereafter switched to the working (ejecting) mode as described previously [10]. The perfusate (recirculating volume of 100 mL) consisted of a modified Krebs-Henseleit solution containing 2.5 mM Ca^{2+}, 11 mM glucose, 1.2 mM palmitate pre-bound to 3% BSA(fatty acid free) and 100 mU/L insulin. Perfusions were performed at a constant workload (preload, 11.5 mm Hg; afterload, 80 mm Hg) and heart rate (paced at 300 beats/min). Heart rate and systolic and diastolic aortic pressures (mm Hg) were measured using a Gould P21 pressure transducer attached to the aortic outflow line. Cardiac output (mL/min) and aortic flow (mL/min) were measured using ultrasonic flow probes (Transonic T206) placed in the left atrial inflow line and the aortic outflow line, respectively. Left ventricular (LV) work (Joules) was calculated as cardiac output x LV

developed pressure (systolic pressure - preload pressure) / 1000 x 0.133 and served as a continuous index of LV mechanical function.

5.2.2 Perfusion Protocol

Hearts were perfused under aerobic conditions in working mode for 15 min prior to stressing by transient ischemia (two 10-min periods of global no-flow ischemia (unpaced) each followed by 5 min reperfusion). Hearts were then perfused aerobically for a further 5-min period, either with vehicle (saline), SB202190 (10 μM), adenosine (500 μM), or SB202190 (10 μM)/adenosine (500 μM), prior to the onset of severe ischemia (30 min of global no-flow ischemia) and reperfusion (30 min). Hearts were frozen for biochemical analyses both at the end of the period of severe ischemia and at the end of reperfusion (Fig. 5-1A).

5.2.3 Immunoblot Analysis of AMPK and p38 MAPK

Heart homogenates were obtained by homogenizing frozen LV tissue in a solution containing 20 mM Tris-HCl (pH 7.4 at 4°C), 50 mM NaCl, 50 mM NaF, 5 mM Na pyrophosphate, 0.25 mM sucrose, protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail (Sigma), and 1 mM dithiothrietol. After homogenization for 30 seconds, protein content of the homogenates was determined using the Bradford protein assay. Samples were diluted and boiled in protein sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose as previously described [24]. Membranes were blocked in 5 % skim milk powder/1 x Tris Buffered Saline containing 0.1% Tween and then immunoblotted with either rabbit anti-phospho-p38 MAPK (Thr180/Tyr182), rabbit anti-p38 MAPK (total), rabbit anti-phospho-AMPK (Thr172), or rabbit anti-AMPK-total (1:1000 dilution) in 5% BSA/1 x TBS. After

extensive washing, the membranes were incubated with a peroxidase-conjugated goat anti-rabbit secondary antibody in 1% skim milk powder/1 x Phosphate Buffered Saline. After further washing, the antibodies were visualized using the Pharmacia Enhanced Chemiluminescence Western Blotting and Detection System. Densitometric analyses of immunoblots (n=3 per experimental group) were performed using Quantity One (4.4.0) Software (Biorad Laboratories). Densitometric values of the phosphorylated proteins are normalized to the total amount of the protein detected.

5.2.4 Measurement of AMPK Activity

The activity of AMPK (nmol/mg protein/min) was measured in 6% polyethylene glycol (PEG) fractions extracted from 200 mg of frozen LV tissue as described previously (25,26). Activity of AMPK in the presence of 5'-AMP (200 μM) was assayed in the 6% PEG fraction by following the incorporation of [γ^{32}P] from [γ^{32}P]ATP into a ser-79 phosphorylation site-specific SAMS peptide (HMRSAMSGLHVKRR), as previously described [26,27].

5.2.5 Measurement of Steady State Rates of Glycolysis and Glucose Oxidation

Glycolysis and glucose oxidation rates were measured directly from the simultaneous production of ^3H_2O (liberated at the enolase step of glycolysis) and $^{14}CO_2$ (liberated at the level of pyruvate dehydrogenase complex and in the citric acid cycle), respectively, from [5-^{3}H]glucose and [U-^{14}C]glucose, as described previously [5]. Perfusate was sampled at pre-determined time points (5, 14, 29, 44, 75, 85, 95, and 105 min), and steady state rates, expressed as μmol [5-^{3}H]glucose or [U-^{14}C]glucose metabolized/g dry wt/min, were calculated.

5.2.6 Calculation of the Rate of Proton Production Arising from Exogenous Glucose Metabolism

When glucose (from endogenous or exogenous sources) is metabolized by glycolysis and subsequently oxidized 1:1, with the associated synthesis and hydrolysis of ATP, the net production of protons is zero. However, if the rate of glycolysis exceeds that of glucose oxidation, there is a net production of two protons per molecule of exogenous glucose that passes through glycolysis, which is not subsequently metabolized. Therefore the rate of proton production attributable to the hydrolysis of ATP arising from exogenous glucose metabolism can be calculated as 2 X (rate of glycolysis – rate of glucose oxidation).

5.2.7 Assay of Glycogen Content and Glucose Uptake

Frozen LV tissue was powdered using a mortar and pestle maintained at the temperature of liquid N_2. Glycogen, in 200 mg of powdered tissue, was converted to glucose by reacting with 4 M H_2SO_4. The amount of glucose (expressed as μmol glucose units/g dry wt) thus obtained was determined using a Sigma glucose analysis kit. The net rate of glycogen synthesis (μmol glucose/min/g dry wt) in hearts during the 30-min reperfusion period was calculated from the increase in [5-^{3}H]- and [^{14}C]glucosyl units in total myocardial glycogen in hearts frozen at the end of reperfusion relative to hearts frozen at the end of severe ischemia. The rate of glucose uptake (μmol/min/g dry wt) during the reperfusion period was calculated as the sum of the net rate of glycogen synthesis and the rate of glycolysis in individual hearts.

5.2.8 Materials

D-[5-^{3}H]glucose and D-[U-^{14}C]glucose were purchased from Dupont Canada Inc., ON, Canada. SB202190 was purchased from Calbiochem, San Diego CA, USA. Adenosine was purchased from Research Biochemicals International, Natrick MA, USA. Anti-phospho-p38 MAPK (Thy180/Tyr182), anti-p38 MAPK (total), anti-phospho-AMPK (Thr172), and anti-AMPK (total) antibodies were obtained from Cell Signaling Technology, Beverly, MA, USA. All other chemicals were reagent grade.

5.2.9 Statistical Analysis

All values are presented as mean ± SE (n observations). The significance of the differences for multiple comparisons between treated and untreated groups was estimated by One-way Analysis of Variance (ANOVA). If significant, selected data sets were compared by Bonferroni's Multiple Comparison Test. Differences were considered significant when $P < 0.05$.

5.3 Results

5.3.1 Left Ventricular Work in Stressed Hearts Prior to and Following Severe Ischemia

LV work (Joules) was stable and similar during the baseline period of perfusion, and in stressed hearts prior to being assigned to vehicle, SB202190, adenosine, or SB202190/adenosine-treated groups as described in the "Materials and Methods" section. All measurable LV work ceased during the periods of transient ischemia, and recovered to similar levels in all groups immediately prior to the onset of severe ischemia (Fig. 5-2A). All measurable LV work ceased during the 30-min period of severe ischemia. During reperfusion, LV work recovered to similar extents in vehicle (0.39±0.12, n=10),

SB202190 (0.25±0.12, n=8), and adenosine-treated (0.30±0.15, n=8) hearts, indicating a loss of the cardioprotective effects of adenosine in stressed hearts (Fig. 5-2B). However, there was a significant improvement in the recovery of LV work in stressed hearts treated with SB202190/adenosine (0.81±0.08, n=7, $P<0.05$), indicating that the p38 MAPK inhibitor restores adenosine-induced cardioprotection in stressed hearts.

5.3.2 Phosphorylation of p38 MAPK and AMPK in Stressed Hearts

The phosphorylation of p38 MAPK (Fig. 5-3A) and AMPK (Fig. 5-3B), and AMPK activity (Fig. 5-3C) was measured in hearts frozen at the end of the 30-min period of severe ischemia (i.e. prior to reperfusion). Values were similar in vehicle, SB202190, adenosine, and SB202190/adenosine-treated hearts at the end of severe ischemia. In hearts frozen at the end of reperfusion, the phosphorylation of p38 MAPK was similar in vehicle, SB202190, and adenosine-treated, but was significantly decreased in hearts treated with SB202190/adenosine (Fig. 5-4A). In parallel, the phosphorylation of AMPK (Fig. 5-4B) and AMPK activity (Fig. 5-4C) were similar in vehicle, SB202190, and adenosine-treated hearts frozen at the end of reperfusion, but were significantly decreased, to non-detectable levels in hearts treated with SB202190/adenosine.

5.3.4 Glucose Metabolism in Stressed Hearts During Reperfusion Following Severe Ischemia

Steady-state rates of glucose metabolism (glycolysis and glucose oxidation) and calculated rates of proton production were assessed to characterize the metabolic consequences of the alterations in p38 MAPK and AMPK phosphorylation in stressed hearts during reperfusion. The rates of glycolysis were similar during reperfusion in vehicle, SB202190, and adenosine-treated hearts where the levels of p38 MAPK and

AMPK phosphorylation were similar. However, in hearts treated with SB202190/adenosine, the rate of glycolysis was decreased by 62% ($P<0.05$) where the phosphorylation of both p38 MAPK and AMPK is attenuated compared with hearts treated with adenosine alone (Fig. 5-5A). The rates of glucose oxidation were similar among the experimental groups during the reperfusion period (Fig. 5-5B). As glycolysis was decreased, and glucose oxidation was unaltered in hearts with attenuated levels of p38 MAPK and AMPK phosphorylation, the calculated rate of proton production from exogenous glucose was concomitantly decreased by 70% ($P<0.05$) compared with hearts treated with adenosine alone (Fig. 5-5C).

5.3.5 Glucose Uptake and Glycogen Metabolism in Stressed Hearts During Reperfusion

The rates of glucose uptake and glycogen metabolism were also calculated in stressed hearts during reperfusion. Despite the marked attenuation of p38 MAPK phosphorylation, as well as AMPK phosphorylation and activity in stressed hearts treated with SB202190/adenosine, the rate of glucose uptake remained unaltered (Fig. 5-6A). In hearts treated with vehicle, SB202190, or adenosine the rates of glycogen synthesis ranged between 6-19% of the rates of glucose uptake. However, in hearts treated with SB202190/adenosine, where the phosphorylation of p38 MAPK and AMPK is attenuated, the rate of glycogen synthesis was approximately 56% of the rate of glucose uptake (Fig. 5-6B). Consequently, myocardial glycogen accumulated to a significantly greater extent in hearts with low levels of p38 MAPK and AMPK phosphorylation (Fig. 5-6C). Furthermore, while the proportions of radiolabelled glycogen (expressed as % total glycogen) in stressed hearts treated with vehicle, SB202190, or adenosine were

67.1±3.7%, 68.2±2.8%, and 63.0±4.1%, respectively, it was significantly greater in hearts treated with SB202190/adenosine (84.4±2.6%), indicative of greater glycogen turnover during reperfusion (Fig. 5-6D). Overall, these data suggest that it is alterations in glucose utilization (i.e. the coordinated balance between glycogen synthesis and glycolysis) that accounts for the decreased rate of calculated H^+ production and restoration of the cardioprotective effects of adenosine in hearts where the phosphorylation of p38 MAPK and AMPK is attenuated.

5.4 Discussion

5.4.1 Summary of Major Findings

This study investigated the roles of p38 MAPK and AMPK in adenosine-induced cardioprotection, and in the regulation of glucose and glycogen metabolism in stressed hearts following severe ischemia. There was a loss of adenosine-induced cardioprotection following severe ischemia in stressed hearts. However, the p38 MAPK inhibitor, SB202190 restored adenosine-induced cardioprotection as evinced by the marked improvement in the recovery of post-ischemic LV work. This was accompanied by an inhibition of p38 MAPK and AMPK phosphorylation, as well as an inhibition of AMPK activity during reperfusion. The inhibition of p38 MAPK and AMPK was associated with an inhibition of the rates of glycolysis and calculated H^+ production. The inhibition of glycolysis occurred independently of changes in glucose uptake, but was accompanied by an elevated rate of glycogen synthesis, and thus an accumulation of myocardial glycogen content. These data indicate that the inhibition of both p38 MAPK and AMPK during reperfusion following severe ischemia is cardioprotective via alterations in glucose metabolism. Rather than being due to changes in glucose uptake,

cardioprotection due to inhibition of p38 MAPK and AMPK was related to an alteration in the partitioning of glucose between glycolysis and glycogen synthesis.

5.4.2 Inhibition of p38 MAPK during Reperfusion is Cardioprotective

The role of p38 MAPK in mediating myocardial IR injury is complex, with studies describing its activation as being both protective and deleterious. Previous reports implicate increased p38 MAPK phosphorylation as an important component of the cardioprotective effects of ischemic preconditioning [15,28]. However, the model utilized in this study is not one of ischemic preconditioning, as the antecedent ischemia protocol fails to enhance the recovery of LV work during reperfusion following severe ischemia; however, the antecedent ischemia protocol produces a model where there is a loss of the cardioprotective effects of adenosine. Interestingly, in this study the level of p38 MAPK phosphorylation is similar between experimental groups at the end of the period of severe ischemia. This may be accounted for by the transient nature of ischemia-induced p38 MAPK activation [29]. In contrast to the above, previous reports also suggest that the activation of p38 MAPK is detrimental during the post-ischemic period, as inhibitors of p38 MAPK reduce apoptosis and infarct size, and improve the recovery of cardiac function [16,30,31]. Although the p38 MAPK inhibitor SB202190 alone did not affect the phosphorylation of p38 MAPK or recovery of LV work during reperfusion, the combination of SB202190/adenosine induced robust cardioprotection and reduced the extent of p38 MAPK phosphorylation at the end of reperfusion, indicating that inhibition of p38 MAPK preserves the cardioprotective effects of adenosine in hearts stressed by antecedent ischemia.

5.4.3 Inhibition of AMPK during Reperfusion is Cardioprotective

AMPK interacts with p38 MAPK to regulate glucose metabolism [12,13]. However, in contrast to the former reports, which suggest p38 MAPK functions downstream of AMPK, our results suggest that it is AMPK that functions downstream of p38 MAPK in the control of myocardial glucose metabolism. Whether AMPK functions to limit myocardial IR injury is uncertain. Previous reports suggest that AMPK plays a protective role in limiting IR injury by promoting glucose uptake, glycolysis, and preventing apoptosis associated with IR injury [21]. In the isolated, fatty acid perfused rat heart the activation of AMPK does indeed accelerate glycolysis; however, it also accelerates the rate of H^+ production [10], which has been demonstrated to impair LV functional recovery during reperfusion [8]. The results presented in this study suggest that the inhibition of AMPK during reperfusion is responsible for marked cardioprotection associated with a partial inhibition of glycolysis and H^+ production, which has been demonstrated to enhance the recovery of LV function during reperfusion following ischemia [5]. The inhibition of glycolysis occurred independently of alterations in glucose uptake, but was accompanied by changes in glycogen metabolism, such that glycogen synthesis was increased [11].

5.4.4 AMPK Regulates Glycogen Metabolism in Stressed Hearts Reperfused Following Severe Ischemia

The role of AMPK in regulating glycogen metabolism is most well studied in skeletal muscle, where there is an inverse relationship between AMPK activity and glycogen content [32,33]. Less well characterized is the role of AMPK in regulating myocardial glycogen metabolism. However, our previous findings have demonstrated

that increased AMPK activity is accompanied by a suppression of the rate of glycogen synthesis, and an acceleration of the rate of glycolysis [10]. Furthermore, we have demonstrated that decreased AMPK activity is accompanied by an increase in the rate of glycogen synthesis and an inhibition of the rate of glycolysis in stressed hearts during aerobic perfusion [11]. In this study, we have extended these findings to a model where stressed hearts are subjected to severe ischemia, where the inhibition of AMPK activity is also accompanied by an increase in the rate of glycogen synthesis and an inhibition of the rate glycolysis, and is associated with cardioprotection during reperfusion following severe ischemia. A greater rate of glycogen synthesis during reperfusion in hearts with low AMPK activity allowed for the accumulation of myocardial glycogen content. Previous reports suggest that glucose liberated from the myocardial glycogen pool is preferentially oxidized [34,35]. This may contribute to the observed cardioprotection in hearts with low AMPK activity by an improved coupling of the rates of glucose oxidation to glycolysis, and thereby decreasing the rate of H^+ production, which has been shown to enhance myocardial functional recovery during reperfusion [5].

5.4.5 Conclusions

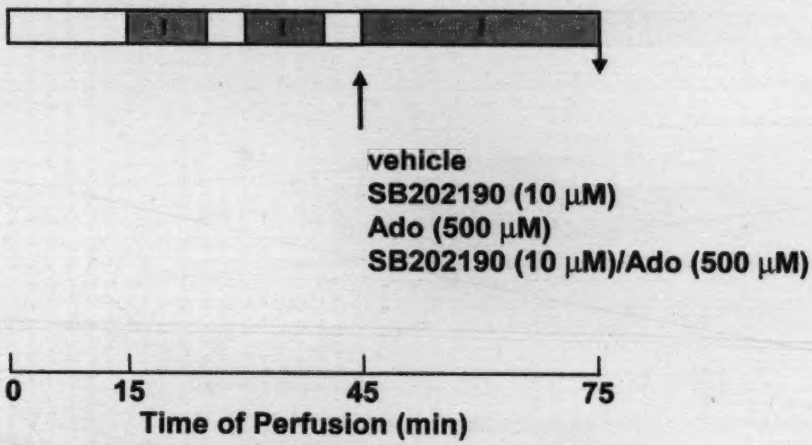
In conclusion this study has shown that the inhibition of p38 MAPK and the subsequent inhibition of AMPK restores adenosine-induced cardioprotection during reperfusion in stressed hearts following severe ischemia. Cardioprotection was associated with an inhibition of the rates of glycolysis and H^+ production that occurred independently of alterations in glucose uptake. The reduction of glycolysis and H^+ production was associated with increased glycogen synthesis and an accumulation of myocardial glycogen content. As the inhibition of p38 MAPK and AMPK reduces the

calculated rate of H^+ production and the subsequent potential for acidosis, the inhibition of either p38 MAPK or AMPK is a novel strategy to restore the protective effects of adenosine in hearts stressed by antecedent ischemia.

Figure 5-1. Experimental protocol for heart perfusions. A, Hearts were perfused aerobically for 15 min prior to stressing by transient ischemia (two 10-min periods of global no-flow ischemia (shaded bars) each followed by 5 min reperfusion), and were treated with either vehicle (saline) (n=6), SB202190 (10 μM) (n=6), adenosine (500 μM) (n=6), or SB202190 (10 μM)/adenosine (500 μM) (n=6) prior to 30 min severe global no flow ischemia. Stressed hearts were then frozen for biochemical analyses at the end of severe ischemia (↓). B, Hearts were perfused in a similar manner as in A, however, following treatment with vehicle (n=10), SB202190 (10 μM) (n=8), adenosine (500 μM) (n=8), or SB202190 (10 μM)/adenosine (500 μM) (n=7) were subjected to 30 min reperfusion following severe ischemia. Hearts were then frozen for biochemical analyses at the end of reperfusion (↓).

Figure 5-1

A. Antecedent Ischemia Protocol – End of Severe Ischemia



B. Antecedent Ischemia Protocol – End of Reperfusion

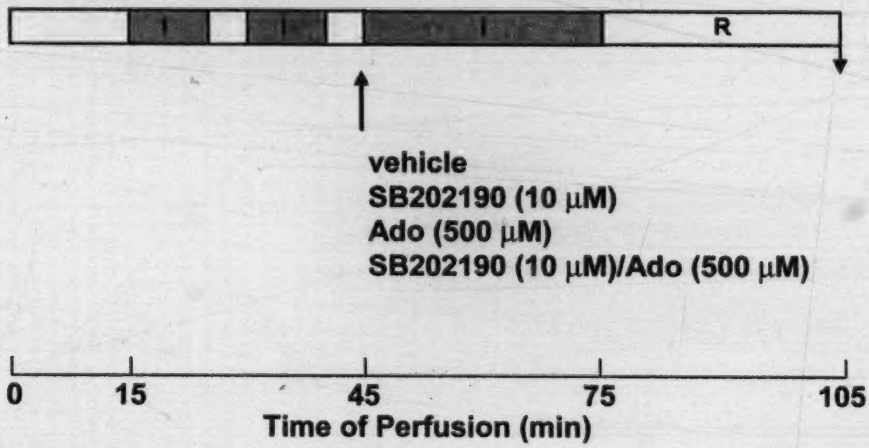


Figure 5-2. Mechanical Function of Stressed Hearts. A, Mechanical function (LV work, mean ± SE) of hearts following transient ischemia and drug treatment, immediately prior to the onset of severe ischemia. B, Mechanical function (LV work, mean ± SE) of hearts during reperfusion following transient ischemia drug treatment, and severe ischemia. The absence or presence of adenosine or SB202190, is indicated by – and + respectively.

Figure 5-2

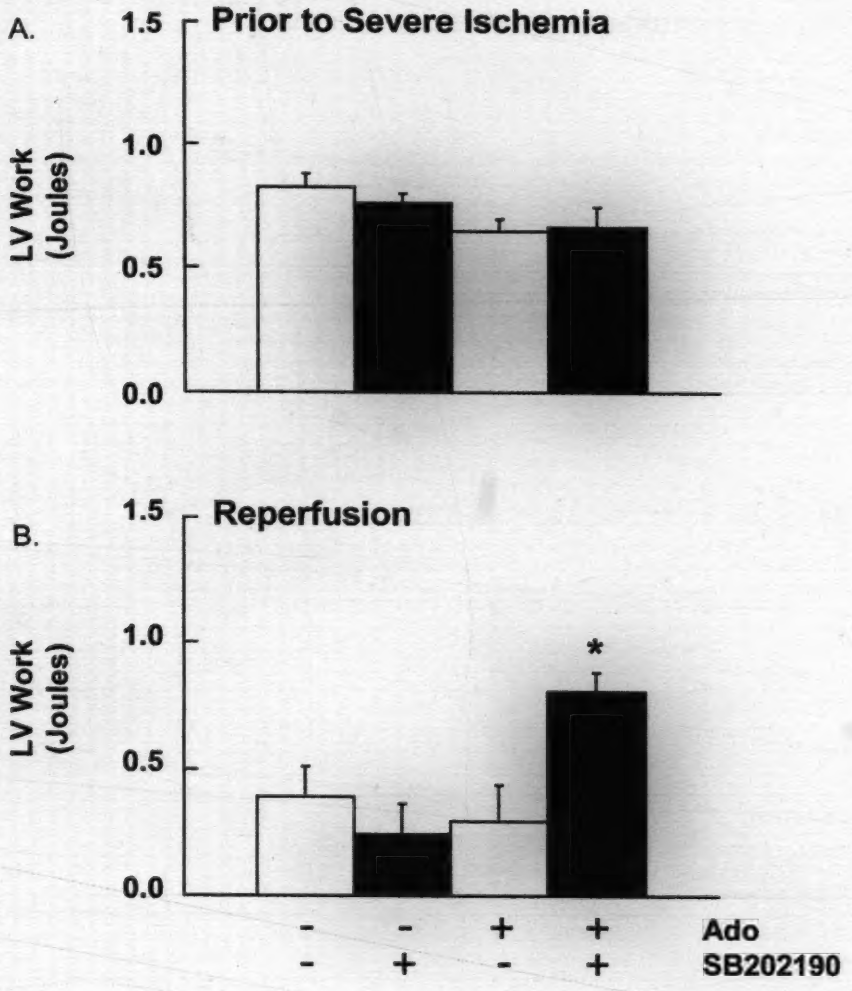

A.
Prior to Severe Ischemia
LV Work (Joules)
1.5
1.0
0.5
0.0
B.
Reperfusion
LV Work (Joules)
1.5
1.0
0.5
0.0
*
- - + + Ado
- + - + SB202190

Figure 5-3. p38 MAPK and AMPK phosphorylation and AMPK Activity in stressed hearts at the end of severe ischemia. Immunoblots and their densitometric analysis (ADU) were measured at the end of severe ischemia as described under "Materials and Methods." A, *Top panel,* representative immunoblots of phosphorylated p38 MAPK and total p38 MAPK from ventricular homogenates. *Bottom panel,* densitometric analysis of immunoblots (n=3 per group). B, *Top panel,* representative immunoblots of phosphorylated AMPK and total AMPK from ventricular homogenates. *Bottom panel,* densitometric analysis of immunoblots (n=3 per group). C, AMPK activity in PEG fractions extracted from ventricular tissue from hearts treated with vehicle, SB202190, adenosine, or SB202190/adensine (n=6 per group). Values represent means ± SE. The absence or presence of adenosine or SB202190, is indicated by – and + respectively.

Figure 5-3

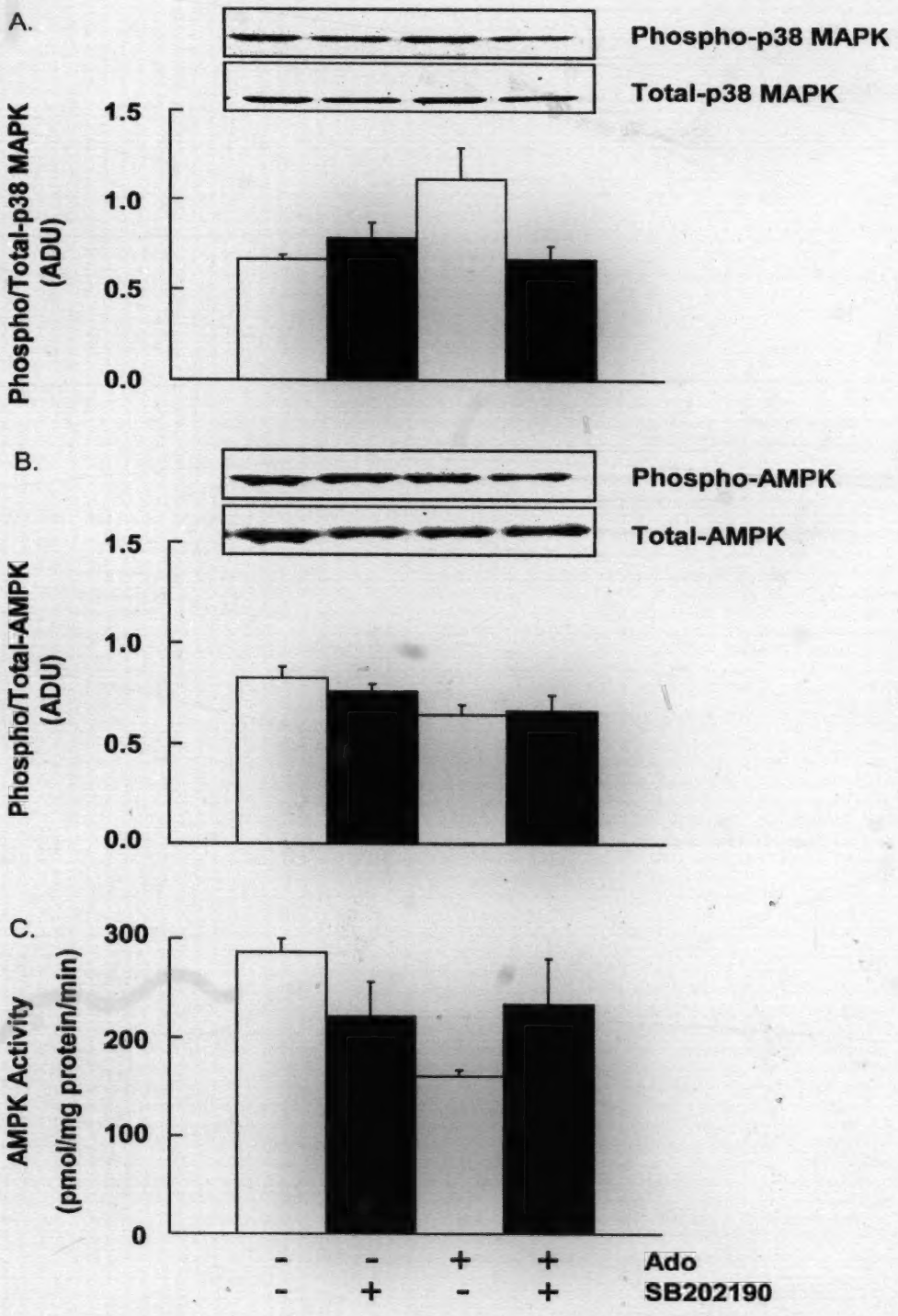

A.
Phospho-p38 MAPK
Total-p38 MAPK
Phospho/Total-p38 MAPK (ADU)
1.5
1.0
0.5
0.0
B.
Phospho-AMPK
Total-AMPK
Phospho/Total-AMPK (ADU)
1.5
1.0
0.5
0.0
C.
AMPK Activity (pmol/mg protein/min)
300
200
100
0
- - + + Ado
- + - + SB202190

Figure 5-4. p38 MAPK and AMPK phosphorylation and AMPK Activity in stressed hearts at the end of reperfusion. Immunoblots and their densitometric analysis (ADU) were measured at the end of reperfusion as described under "Materials and Methods." A, *Top panel,* representative immunoblots of phosphorylated p38 MAPK and total p38 MAPK from ventricular homogenates. *Bottom panel,* densitometric analysis of immunoblots (n=3 per group). B, *Top panel,* representative immunoblots of phosphorylated AMPK and total AMPK from ventricular homogenates. *Bottom panel,* densitometric analysis of immunoblots (n=3 per group). C, AMPK activity in PEG fractions extracted from ventricular tissue from hearts treated with vehicle, SB202190, adenosine, or SB202190/adensine (n=6 per group). Values represent means ± SE. The absence or presence of adenosine or SB202190, is indicated by – and + respectively. * indicates a significant difference from adenosine-treated hearts.

Figure 5-4

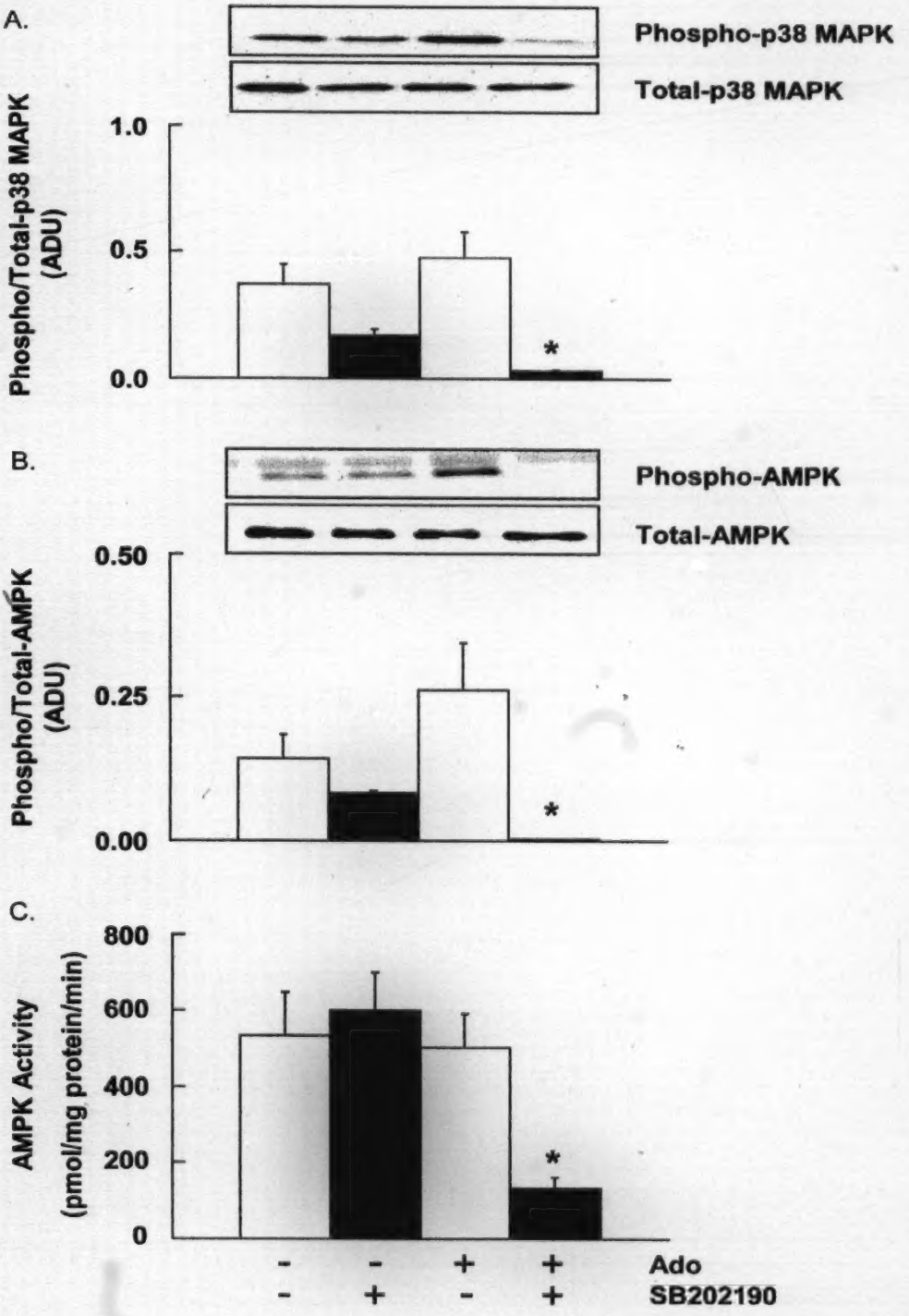

A.
Phospho-p38 MAPK
Total-p38 MAPK
Phospho/Total-p38 MAPK (ADU)
1.0
0.5
0.0
*
B.
Phospho-AMPK
Total-AMPK
Phospho/Total-AMPK (ADU)
0.50
0.25
0.00
*
C.
AMPK Activity (pmol/mg protein/min)
800
600
400
200
0
*
- - + + Ado
- + - + SB202190

Figure 5-5. Rates of glucose metabolism and calculated proton production in stressed hearts during reperfusion following severe ischemia. Glycolysis (A), glucose oxidation (B) and the calculated rate of proton production from glucose metabolism (C) were assessed as described under "Materials and Methods" for hearts treated with vehicle (n=7), SB202190 (n=8), adenosine (n=7), or SB202190/adenosine (n=7). Values (μmol/g dry wt/min) represent means ± SE. The absence or presence of adenosine or SB202190, is indicated by – and + respectively. * indicates a significant difference from adenosine-treated hearts.

Figure 5-5

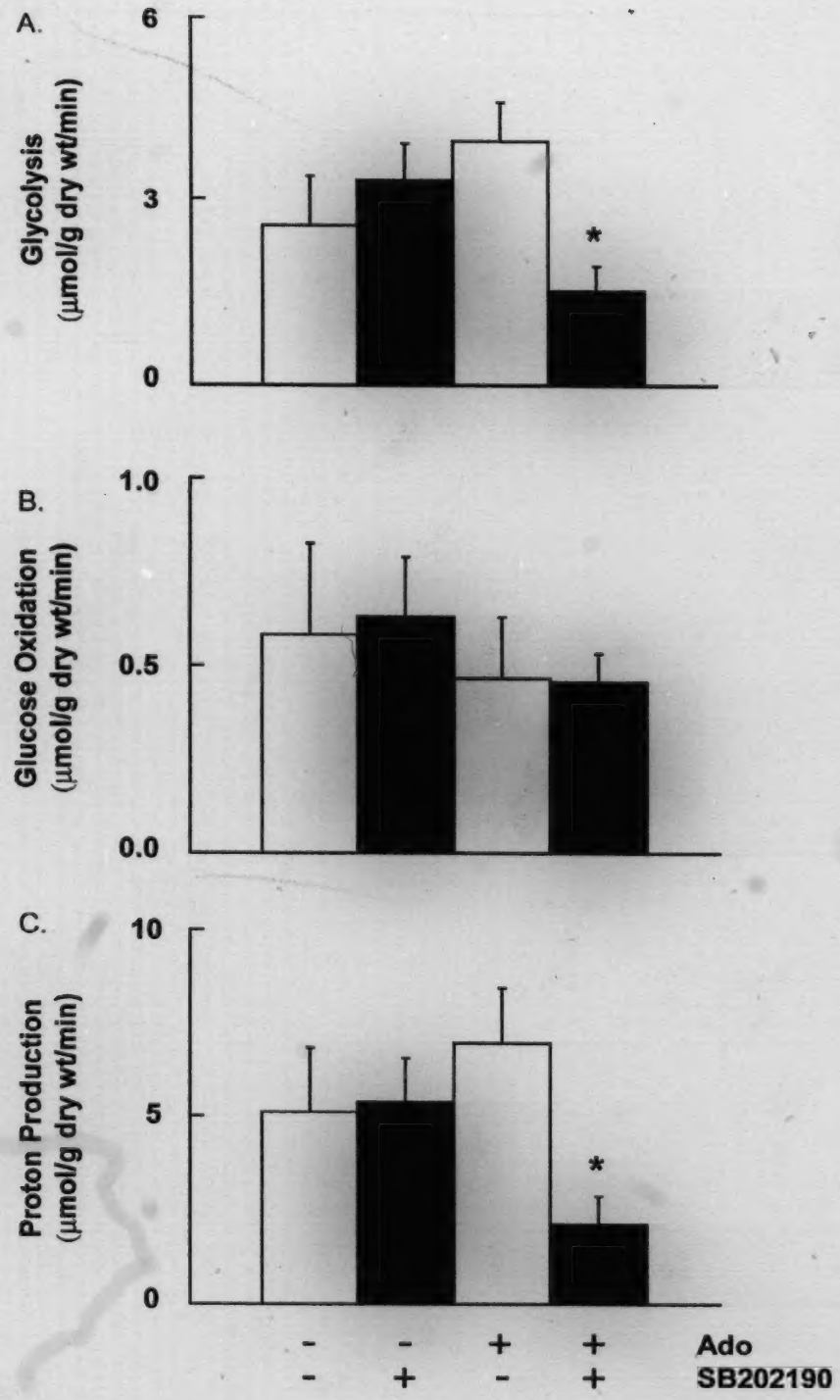
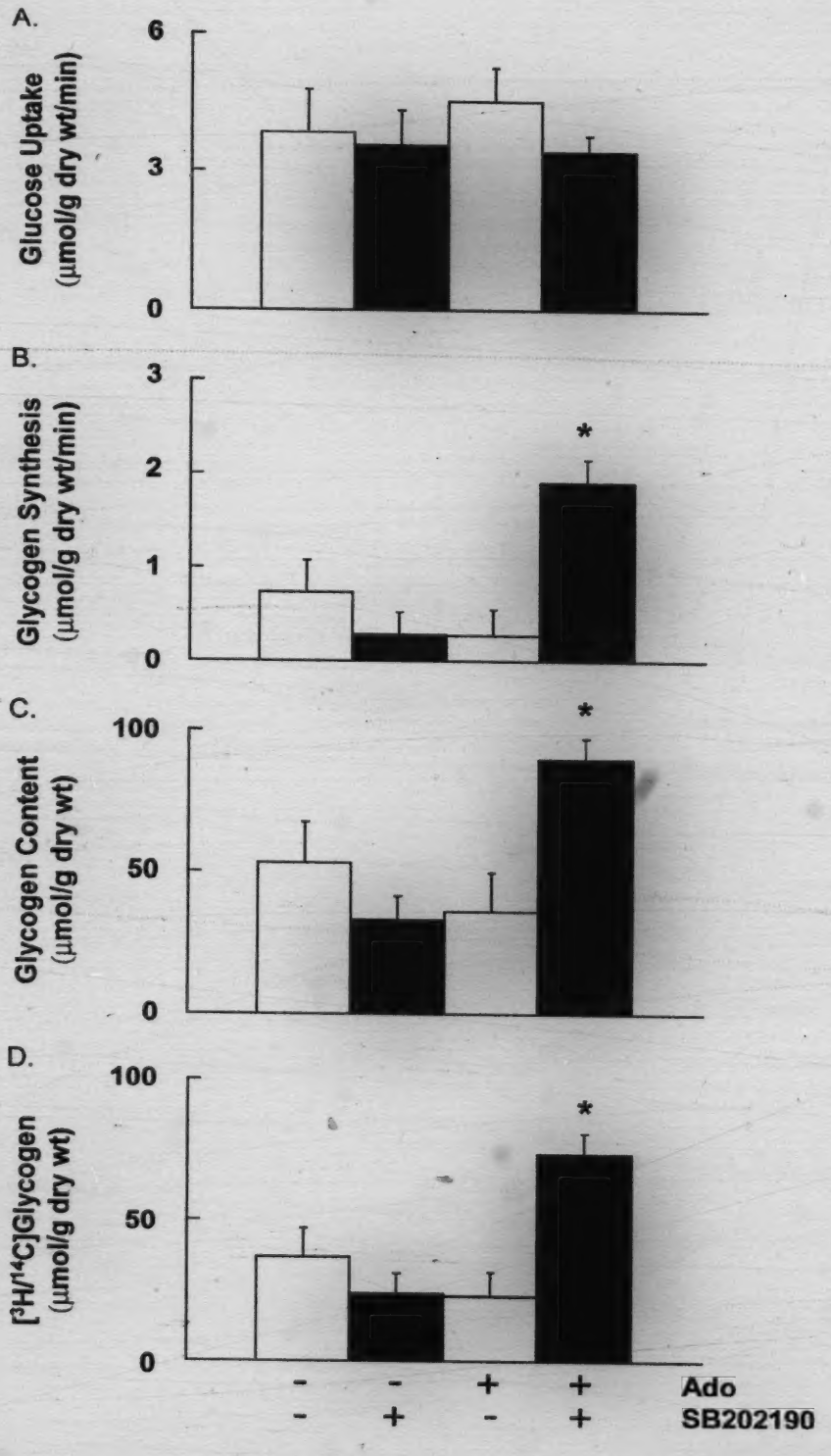

A.
Glycolysis
(μmol/g dry wt/min)
6
3
0
*
B.
Glucose Oxidation
(μmol/g dry wt/min)
1.0
0.5
0.0
C.
Proton Production
(μmol/g dry wt/min)
10
5
0
*
- - + + Ado
- + - + SB202190

Figure 5-6. Glucose uptake and glycogen metabolism in stressed hearts during reperfusion following severe ischemia. Glucose uptake (A), glycogen synthesis (B), total glycogen content (C), and $^{3}H/^{14}C$ glycogen content (D) were assessed as described under "Materials and Methods" for hearts treated with vehicle, SB202190, adenosine, or SB202190/adenosine (n=6 per group). Values (μmol/g dry wt/min for A and B and μmol/g dry wt for C and D) represent means ± SE. * indicates a significant difference from adenosine-treated hearts.

Figure 5-6


A.
Glucose Uptake
(μmol/g dry wt/min)
6
3
0
B.
Glycogen Synthesis
(μmol/g dry wt/min)
3
2
1
0
*
C.
Glycogen Content
(μmol/g dry wt)
100
50
0
*
D.
[³H/¹⁴C]Glycogen
(μmol/g dry wt)
100
50
0
*
- - + + Ado
- + - + SB202190

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Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS

Experimental Model and Methods

6.1 The Isolated Working Rat Heart Preparation

The isolated working rat heart preparation represents a highly reproducible experimental model that allows for the assessment of a wide variety of biochemical, physiological, morphological, and pharmacological indices. This experimental model provides a distinct advantage over the use of isolated muscle preparations where the reliance on superfusion may be insufficient for the adequate provision of oxygen and nutrients the central core of the muscle, thereby producing an experimental scenario that does not lend itself readily to the study of metabolism. The isolated working heart preparation also provides a distinct advantage over the use of isolated cell preparations, as these preparations are not ideal for the study of energy substrate metabolism due to their quiescent nature and thus non-physiological energy demand.

The studies undertaken in this thesis utilized perfusion with crystalloid solution, rather than blood. Although perfusion with crystalloid solution results in relatively high coronary flow values due to the low O_2 carrying capacity, the crystalloid solution is nonetheless capable of delivering adequate amounts of O_2. This is attributed to the high partial pressure (p) of O_2 obtained by gassing the solution with a mixture of 95% O_2 and 5% CO_2. In fact gassing with mixtures containing only 70% O_2 is still sufficient to achieve an adequate pO_2, as isolated heart preparations perfused in this manner are still able to respond to inotropic agents for sustained periods of time without incurring injury [1].

The isolated working rat heart is an ideal experimental model for the simultaneous measurement of cardiac function and cardiac energy substrate metabolism.

A major factor controlling flux through the pathways of fatty acid β-oxidation, glycolysis, glucose oxidation, and the TCA cycle is the external work being performed by the heart. The ability to perfuse the isolated heart at various levels of preload and afterload, as well as with both glucose and fatty acids as energy substrates allows the characterization of cardiac substrate metabolism at physiologically relevant workloads in the absence of confounding effects of other organs, and systemic circulating neurohormonal factors.

6.2 Transient Ischemia as a Clinically Relevant Experimental Model

A common criticism of a number of studies using the isolated heart preparation as a model of ischemic heart disease is that most studies rely on an extremely simplistic form of ischemia applied in otherwise "healthy" hearts. Clinically, the manifestations of ischemic heart disease develop gradually, in the face of ongoing physiological adaptations to the underlying pathopysiology, and thus experimental results may not necessarily be readily extrapolated to the clinical setting and vice versa. The studies undertaken in Chapters 3, 4, and 5 of this thesis involved the use of isolated hearts stressed by transient ischemia. Although the transient ischemia protocol itself is quite simplistic, it nonetheless recapitulates the clinical findings of reduced cardioprotective effectiveness of adenosine and adenosine mimetic agents as described in the relevant chapters of the Results section. Thus, the model of transient ischemia is ideal for the investigation of the possible mechanisms underlying the poor cardioprotective effects of adenosine, and adenosine mimetics in the clinical setting. As such, this model was exploited to characterize the role of stress-responsive protein kinases as well as energy substrate metabolism in the cadioprotective effects of adenosine.

6.3 Direct Measurement of Energy Substrate Metabolism

The direct measurement of energy substrate metabolism has been performed using radioisotopes labeled at specific hydrogen and carbon atoms to quantify the rate of flux through the pathways of fatty acid β-oxidation, glycolysis, and glucose oxidation using [9,10-^{3}H]palmitate, [5-^{3}H]glucose, and [U-^{14}C]glucose respectively. Details regarding the quantitative collection of metabolic by-products ($^{3}H_2O$ and $^{14}CO_2$) are described within each of the results chapters.

6.3.1 Direct Measurement of Glucose Metabolism

The use of dual labeled glucose ([5-^{3}H]glucose and [U-^{14}C]glucose) provides the distinct advantage in that it allows the simultaneous measurement of both glycolysis and glucose oxidation in a single heart. Glycolysis can also be measured using [2-^{3}H]glucose, however, the use of [2-^{3}H]glucose may have several limitations. The ^{3}H label at the 2-position can be subject to intra-molecular exchange at the 1-position. As the rate of glycolysis using this tracer depends on the rate of release of $^{3}H_2O$, liberated at the phosphoglucose isomerase step of glycolysis, intra-molecular exchange of the label can lead to an underestimation of the actual rate of glycolysis. Conversely, the rate of glycolysis can also be overestimated using [2-^{3}H]glucose. The fractional velocity (V_{max}) of GAPDH can be significantly inhibited following IR [2]. As $^{3}H_2O$ is liberated from [2-^{3}H]glucose upstream of GAPDH, restriction of flux at the level of GAPDH can result in an overestimation of the actual rate of glycolysis. [5-^{3}H]glucose circumvents the above mentioned drawbacks. As $^{3}H_2O$ is liberated from [5-^{3}H]glucose downstream of GAPDH, at the enolase step of glycolysis, the restriction of flux at the level of GAPDH does not introduce errors into the measurement of flux through glycolysis using this tracer. As

the rates of glycolysis and glucose oxidation are directly and accurately calculated, the degree of uncoupling between the two processes is also accurately calculated.

6.4 Proton Production as an Index of Uncoupling Between Glycolysis and Glucose Oxidation

The release of lactate provides an indirect measure of the degree of uncoupling between the processes of glycolysis and glucose oxidation as lactate release depends not only on production by anaerobic glycolysis (glucose + 2 Pi + 2 ADP $\rightarrow$ 2 lactate + 2 ATP + 2 H_2O), but also on consumption by mitochondrial oxidation. Thus the simultaneous release and production of lactate complicates the use of this parameter as an index of uncoupling between glycolysis and glucose oxidation.

In the studies described in this thesis, H^+ production from the catabolism of glucose was used as an index of uncoupling between the rates of glycolysis (measured directly using [5-^{3}H]glucose) and glucose oxidation (measured directly using [U-^{14}C]glucose). As H^+ production is calculated from the hydrolysis of glycolytically derived ATP, any increase in the rate of glycolysis such that it is greater than the rate of glucose oxidation, allows the calculation of the rate of H^+ production as summarized:

Glycolysis:

$$\text{glucose} + 2\ \text{ADP} + 2\text{P}_\text{i} + 2\ \text{NAD}^+ \rightarrow 2\ \text{pyruvate} + \mathbf{2\ ATP} + 2\ \text{NADH} + 2\ \text{H}_2\text{O} + 2\ \text{H}^+$$

Glucose Oxidation:

$$\text{glucose} + 38\ \text{ADP} + 38\ \text{P}_\text{i} + 38\ \text{H}^+ + 6\ \text{O}_2 \rightarrow 6\ \text{CO}_2 + 38\ \text{ATP} + 42\ \text{H}_2\text{O}$$

When the net rate of ATP hydrolysis is greater than the rate of mitochondrial ATP production (glucose oxidation), glycolytically derived ATP is hydrolyzed yielding H^+ as summarized:

Proton Production:

$$\mathbf{2\ ATP} + 2\ H_2O \rightarrow 2\ ADP + 2P_i + \mathbf{2\ H^+}$$

Therefore, this approach provides a direct and accurate index of the degree of uncoupling between glycolysis and glucose oxidation, which appears to be a critical component that influences adenosine-induced cardioprotection.

6.5 Glucose Uptake

Glucose uptake, distinguished from glucose transport as it is dependent upon the intracellular fates of glucose was calculated as the sum of the rates of glycolysis and net glycogen synthesis. Although glucose uptake can be assessed through the use of "non-metabolisable" glucose analogues such as 2-deoxyglucose, several limitations must be considered. Kinetic differences in the Michaelis Constant (K_M) and V_{max} values for trans-membrane flux (transport) and for the rate of phosphorylation (uptake) between 2-deoxyglucose and glucose require the use of a correction factor termed the Lumped Constant (LC), where LC is the ratio of 2-deoxyglucose uptake over the rate of glucose uptake. Although this ratio has previously been considered to be constant in heart muscle, recent reports demonstrate changes in the LC associated with changes in metabolic environment, whrere the presence of insulin stimulates glucose uptake to a greater extent than 2-deoxyglucose uptake [3]. Furthermore, 2-deoxyglucose-6-phosphate is also a substrate for glycogen synthase, as a result to calculate the total index of glucose uptake using this method requires determining the extent of 2-deoxyglucose incorporation into the myocardial glycogen pool [3]. Moreover still, 2-deoxyglucose-6-phosphate may inhibit the actual rate of glycolysis, thereby interfering with rates of glucose metabolism with probable effects on mechanical and metabolic function of the

working heart. As the approach to measure glucose uptake in this thesis takes into account the two intracellular fates of glucose, i.e. catabolism via glycolysis and incorporation into the myocardial glycogen pool, this approach is ideally suited for the working rat heart where glucose uptake and metabolism influence LV mechanical function.

Cardioprotection During and Following Hypothermic Ischemia

6.6 ERK Signaling

The severity of myocardial IR injury progressively increases as does the duration of ischemia. Hypothermic cardioplegic arrest is the gold standard for delaying and minimizing myocardial IR injury during cardiac surgery or transplantation. It is generally agreed upon that the protective effects of hypothermic cardioplegia are due to the arrest of cardiac contractile activity, and thus marked attenuation of metabolic requirements. The modest protective effect of PD98059 during the phase of hypothermic storage indicates that this phase may be a relatively important period for ERK 1/2 related strategies of cardioprotection. Interestingly, inhibition of the ERK cascade by PD98059 has been demonstrated to confer cytoprotection following rewarming from hypothermia [4], however, PD98059 was unable to confer cardioprotection when present during the phase of rewarming. This likely suggests a protective role of ERK activation during reperfusion as reported previously [5,6].

6.7 p38 MAPK Signaling

Similar to previous reports, the results in Chapter 2 suggest a clinical utility for the inhibition of p38 MAPK in experimental models of heart transplantation [7]. However,

the timing of p38 MAPK inhibitor administration is paramount. The p38 MAPK inhibitor FR167153 is effective as an additive to Celsior solution. The p38 MAPK inhibitor SB202190 was not added to St. Thomas's II solution during the period of hypothermic ischemia, however, was present either during the phase of Langendorff rewarming, or working mode reperfusion.

The lack of functional improvement by inhibition of p38 MAPK during the initial phase of rewarming is intriguing. Interestingly, oxidative stress induced by hydrogen peroxide (H_2O_2) transiently activates p38 MAPK and increases the phosphorylation of its downstream targets, MAPKAPK2 and heat shock protein 27 (HSP27) [8], which appears to be required for the tranlocation of HSP27 to the cardiac sarcomere and subsequent cardioprotection [9]. Although speculative, it is possible that the likely, burst of ROS during the rewarming phase is required to transiently activate p38 MAPK, and promote the translocation of HSP27 to the cardiac sarcomere to confer cardioprotection, however, this was not addressed in these studies.

Conversely, the sustained activation of p38 MAPK during the phase of working mode reperfusion is deleterious, as evinced by the marked cardioprotective effects of SB202190 during this phase. This finding is in line with previous reports; however, the specific mechanisms underlying this cardioprotection remain to be resolved. Although the inhibition of apoptosis, and decreased myocardial inflammatory mediator generation are implicated in the cardioprotective effects of p38 MAPK inhibitors, the rapid functional benefits of SB202190 during the working phase of reperfusion suggest non-genomic mechanisms of cardioproteciton. Recently, alterations in the subcellular compartmentalization of p38 MAPK have been suggested to contribute to the protective

effects of acute adenosine-induced preconditioning. Preconditioning induced by the adenosine A_1 receptor agonist, AMP-579 by increases the phosphorylation and activation of p38 MAPK in the nuclear/myofilament fraction, whereas myocardial IR increases p38 MAPK phosphorylation in the cytosolic, membrane, and mitochondrial fractions [10]. Although the subcellular compartmentalization of p38 MAPK was not addressed in this thesis, it is tempting to speculate that alterations in compartmentalization of p38 MAPK contribute to the profound cardioprotective effects of SB202190 during the working phase of reperfusion, following cardioplegic arrest and prolonged hypothermic ischemia.

Regulation of Myocardial Energy Substrate Metabolism in the Stressed Heart

6.8 AMPK and Energy Substrate Metabolism

The studies described in Chapters 3, 4, and 5 of this thesis examined the role of AMPK in regulating both palmitate and glucose metabolism in hearts stressed by transient ischemia in response to adenosine treatment. In contrast to previous studies, the activation of AMPK was found not to influence the rates of myocardial palmitate oxidation, or glucose uptake, but rather alter the balance between glycogen synthesis and glycolysis, such that glycolysis is favored.

The lack of effect of AMPK activation on the rates of palmitate oxidation and glucose uptake is particularly surprising. The dissociation between myocardial fatty acid oxidation and AMPK activity has been reported previously [11,12]. Of interest is the observation that the majority of studies reporting increased glucose uptake in response to AMPK activation do so in fast-twitch skeletal muscle, whereas glucose uptake is unaltered in slow-twitch skeletal muscle despite the activation of AMPK [13,14]. These

results suggest that the role of AMPK in skeletal muscle is dependent on fiber type. The absence of increased myocardial glucose uptake despite the activation of AMPK further supports the fiber-type dependence for it ability to regulate glucose uptake.

The majority of myocardial glucose uptake is accounted for by the glycolytic pathway, and the manifestations of elevated AMPK activity occur at the level of balance between glycolysis and glycogen synthesis. Elevated AMPK activity, in response to adenosine treatment in the stressed heart inhibits net glycogen synthesis, thereby shuttling available glucose towards glycolysis, thus elevating H^+ production attributable to the hydrolysis of glycolytic ATP. These findings are in line with previous reports describing the ability of AMPK to inhibit ATP consumption (glycogen synthesis), while promoting ATP generation (glycolysis). Although these studies did not determine whether it is increase in glycolysis that decreases glycogen synthesis, or the decrease in glycogen synthesis that increases glycolysis, the fact that both processes are occurring simultaneously suggests that both glycolysis and glycogen synthesis are regulated in a coordinate manner. Further evidence for this coordinate regulation is provided by studies with p38 MAPK inhibitors, which prevent the adenosine-induced elevation of AMPK, glycolysis, and H^+ production, by relieving the inhibition of net glycogen synthesis.

6.9 Regulation of Myocardial Glucose Utilization by p38 MAPK and AMPK

Although previous reports have demonstrated that p38 MAPK and AMPK form a signal transduction cascade, with AMPK being upstream of p38 MAPK, the results presented in this thesis indicate that in the isolated rat heart, p38 MAPK is upstream of AMPK, and that p38 MAPK regulates glycolysis and glycogen synthesis via AMPK. This discrepancy may be related to species differences. For example, in the isolated

mouse heart, where MEK 3/6 has been deleted, AMPK facilitates the recruitment of p38 MAPK to TAB1, where p38 MAPK subsequently undergoes autophosphorylation [15], however, TAB1 protein appears to absent from isolated rat cardiac myocytes [16]. Although the studies undertaken in this thesis do not delineate the nature by which AMPK is activated by p38 MAPK (i.e. direct phosphorylation or by other indirect mediators) it is of interest that the protein sequences of both AMPK α-subunits (α_1 and α_2) contain the consensus sequence for phosphorylation by p38 MAPK. Although the critical threonine-172 residue is not a part of the p38 MAPK consensus sequences found in the α_1 and α_2 subunits of AMPK, it may be that the p38 MAPK consensus sequences are involved in the allosteric regulation of threonine-172 phosphorylation.

The ability of the selective p38 MAPK inhibitors, SB202190 and SB203580 to prevent the adenosine-induced phosphorylation of both p38 MAPK and AMPK, revealed that p38 MAPK is major regulator of myocardial glucose utilization. Furthermore, with the lack of selective AMPK inhibitors, both SB202190 and SB203580 allowed the assessment of myocardial energy substrate metabolism in the face of alterations in the phosphorylation of AMPK. These studies revealed a strong inverse correlation between the rates of glycogen synthesis and AMPK activity, as well as AMPK activity and glycogen content. Interestingly, glycogen degradation was not altered by differing levels of AMPK phosphorylation. These alterations in glycogen metabolism were closely associated with changes in glycolysis and H^+ production, such that glycolysis and H^+ production were significantly attenuated when the activity of AMPK was attenuated in the face of near physiological levels of myocardial glycogen content. These alterations in glucose utilization highlight a dynamic balance between the rates of glycolysis and

glycogen synthesis, as well as between glycogen content and glycolysis which are important in determining the extent of uncoupling between glycolysis and glucose oxidation. The extent of coupling between glycolysis and glucose oxidation itself appears to be a critical mediator of adenosine-induced cardioprotection.

6.10 Glycolysis, Glycogen Synthesis and Cardioprotection

The salutary alterations in glucose metabolism in response to treatment with a combination of the p38 MAPK inhibitor, SB202190 and adenosine persist in stressed hearts following severe global ischemia. Specifically, decreased phosphorylation of p38 MAPK and AMPK is accompanied by a reduction in the rates of glycolysis and H^+ production, as well as an increased rate of glycogen synthesis and increased myocardial glycogen content. This suggests that improved metabolic handling of glucose has the capacity to exert cardioprotection, and is not merely a consequence associated with improved LV function. Furthermore, it points to the dynamic balance between glycolysis and glycogen synthesis, which is regulated by p38 MAPK and AMPK as being a potentially viable target to limit the deleterious effects of myocardial IR injury. By promoting glycogen synthesis, potentially excessive rates of glycolysis can be attenuated, thereby reducing the injurious effects of acidosis.

The role glycolysis during reperfusion, following ischemia is controversial. Indeed there are studies demonstrating that increased glycolysis during ischemia improves the recovery of function due to the increase in glycolytic ATP production [17]. Furthermore, there are also reports demonstrating that inhibition of glycolysis during reperfusion is detrimental [18]. This has been attributed to the importance of glycolytic ATP production for maintaining sarcolemmal ion pumps [19]. On the contrary, the

results presented in Chapter 5 of this thesis suggest that attenuation of the rate of glycolysis during the period of reperfusion following ischemia imparts cardioprotection. Inherent differences in choice of experimental model (working rat heart preparation versus Langendorff rat heart [17] and Langendorff rabbit [18] heart preparation, respectively), and differences in perfusate composition (inclusion of 1.2 mM palmitate versus fatty acid free perfusate [17,18]) distinguish the results described in this thesis from the above cited, and in part may account for differences with regards to the role of glycolysis during reperfusion.

During reperfusion following ischemia, in the absence of fatty acids the heart must increasingly rely on glycolysis, and subsequent glucose oxidation to derive ATP in order to meet energy requirements and re-establish ionic homeostasis. However, in the presence of fatty acids, glucose metabolism is no longer the sole energy producing pathway. Furthermore, the rate of fatty acid oxidation and oxidative ATP production recovers rapidly in the post-ischemic period, while contractile function remains depressed [20,21,22]. The rapid recovery of fatty acid oxidation can suppress glucose oxidation, ultimately uncoupling glycolysis and glucose oxidation, and thus enhancing the potential for H^+ production from glucose metabolism. By partially inhibiting the rate of glycolysis in the fatty acid perfused heart during the post-ischemic period, non-oxidative ATP production and hydrolysis is reduced. Thus, inhibition of glycolysis causes and marked decrease in the calculated rate of H^+ production from exogenous glucose metabolism. This reduction in the potential for acidosis has previously been demonstrated to be cardioprotective [23,24], and the results obtained in this thesis further support this observation. Therefore, whether glycolysis is cardioprotective or detrimental during

reperfusion following ischemia is profoundly influenced and regulated by the energy substrates provided during the post-ischemic period, such that in the fatty acid perfused heart, inhibition of glycolysis appears to be beneficial.

Conclusions

In conclusion, the studies described in thesis indicate that the inhibition of stress-responsive protein kinases has potential clinical utility in the management of ischemic heart disease. Studies demonstrating that the inhibition of p38 MAPK improves the recovery of LV work following cardioplegic arrest and hypothermic ischemia indicate that the p38 MAPK is a major mediator of myocardial IR injury, and further studies of p38 MAPK inhibitors in experimental models of heart transplantation are warranted. Studies in hearts stressed by transient ischemia indicate that the activation of AMPK, downstream of p38 MAPK underlies the adenosine-induced acceleration of the rates of glycolysis and H^+ production by altering the partitioning of glucose between glycolysis and glycogen synthesis, independent of changes in glucose uptake. Furthermore, the ability of p38 MAPK inhibitors to prevent the adenosine-activation of p38 MAPK and AMPK revealed significant inverse relationships between glycogen synthesis and AMPK activity, as well as AMPK activity and glycogen content. These inverse relationships revealed a dynamic balance between the rates of glycolysis and glycogen synthesis, which results in increased H^+ when glycolysis is favored, and decreased H^+ production when glycogen synthesis is favored. This dynamic balance between glycolysis and glycogen synthesis, and its regulation by p38 MAPK and AMPK persists in stressed hearts following severe ischemia. As inhibition of p38 MAPK and AMPK reduces the

rate of H^+ production and the subsequent potential for acidosis, the inhibition of either p38 MAPK or AMPK is a novel strategy to restore the protective effects of adenosine in hearts stressed by antecedent ischemia. Thus, the stress-responsive protein kinases, p38 MAPK and AMPK represent novel, and viable pharmacological targets that can be exploited to manipulate myocardial energy substrate metabolism in order to limit IR injury.

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Chapter 7

FUTURE DIRECTIONS

Experimental Models of Cardiac Transplantation

7.1 Cardioplegia and Hypothermic Ischemia

The ability of both PD98059 and SB202190 to improve the recovery of mechanical function following cardioplegic arrest and prolonged hypothermic ischemia suggests that both ERK and p38 MAPK signaling cascades are involved in the regulation of IR injury. As the beneficial effects of ERK and p38 MAPK inhibition were observed during differing phases of the perfusion protocol (hypothermic ischemia and working-mode reperfusion, respectively), it is warranted to characterize the possible additive or synergistic effects of PD98059 and SB202190 in a similar experimental model. Supplementing the cardioplegic solution employed during the period of hypothermic ischemia with PD98059 may limit hypothermic injury, and thus impart therapeutic benefit during cold storage. Exposing hearts to SB202190 during the phase of working reperfusion may limit myocardial IR injury, and thus impart enhanced cardioprotection than either agent alone. These experiments would potentially further the ongoing refinements in tissue preservation, and improve cardiac allograft viability.

Cardiac Energy Substrate Metabolism, Ionic Homeostasis, and Efficiency

7.2 Coupling Between Glycolysis and Glucose Oxidation and its Influence on Cardiac Ionic Homeostasis

The studies described in Chapters 3, 4, and 5 demonstrate differing extents of coupling between glycolysis and glucose oxidation, and thus differing degrees of H^+ production. Although Chapter 5 convincingly demonstrates that a reduction in the rate of

H^+ production is cardioprotective during reperfusion following severe ischemia, the mechanisms underlying this cardioprotection require further characterization. The improved coupling between glycolysis and glucose oxidation may have salutary effects on cardiac ionic homeostasis, specifically Na^+ and Ca^{2+} homeostasis that contribute to cardioprotection.

The ability to measure intracellular Na^+ and Ca^{2+} concentrations in the isolated rat heart using the fluorescence probes sodium benzofuran isophthalate (SBFI) [1] and indo-1-acetoxymethyl ester (indo-1-AM) [2], respectively, allows the contribution of alterations in Na^+ and Ca^{2+} homeostasis to ischemic injury to be characterized. As measurement of intracellular Na^+ and Ca^{2+} with SBFI and indo-1-AM involves the collection of spectra at different excitation (SBFI – 380 nm, indo-1-AM – 354 nm) and emission (SBFI – 530 nm, indo-1-AM – 405 nm ad 485 nm) wavelengths, it is theoretically possible to characterize the rates of glycolysis, glucose oxidation, and H^+ production, as well as intracellular Na^+ and Ca^{2+} concentration in the same heart. Use of the transient ischemia model described in Chapters 3 and 4, and the antecedent ischemia model described in Chapter 5 would permit the delineation of the roles of p38 MAPK and AMPK in the regulation of ionic homeostasis and ischemic injury via alterations in cardiac energy metabolism [3].

7.3 Effects of p38 MAPK and AMPK on Cardiac Efficiency

As the results presented in Chapter 3 suggest that the activation of AMPK, following transient ischemia alters glucose utilization independently of changes in palmitate oxidation, it is unlikely that AMPK activation in this model significantly affects cardiac efficiency at the level of O_2 consumption. However, the results presented in

Chapter 5, where an improved coupling between glycolysis and glucose oxidation was accompanied by an improved recovery of myocardial function following severe ischemia, suggests that ATP hydrolysis is more efficiently converted to contractile work in hearts where the rate of H^+ production is reduced. This may be related to a lesser amount of ATP hydrolysis required to correct disturbances in ionic homeostasis. Insight into this facet of cardiac efficiency, in the model of antecedent ischemia may also be gained by measuring intracellular Na^+ and Ca^{2+} concentrations as described above.

7.4 Glycogen Metabolism and Cardioprotection

The findings in Chapter 5 indicate that optimizing energy substrate metabolism, particularly at the level of glycolysis and H^+ production via the pharmacological inhibition of p38 MAPK, or AMPK in adenosine-treated stressed hearts is cardioprotective. The coupling of glycolysis to glucose oxidation is an important determinant of cardioprotection, and glycogen metabolism is itself an important component of overall glucose utilization. However, as this study measured total glycolysis and total glucose oxidation, it cannot ascribe the improved coupling between glycolysis and glucose oxidation to the preferential oxidation of glucose liberated from the myocardial glycogen pool (endogenous glucose) [4]. Labeling the myocardial glycogen pool employing a modified pulse-chase protocol using [5-^{3}H]glucose and [U-^{14}C]glucose would allow the contribution of endogenous glucose to total glycolysis and total glucose oxidation to be delineated. Such studies would aid in further clarifying the roles of both p38 MAPK and AMPK in the regulation of myocardial glycolysis, glucose oxidation, H^+ production, as well as glycogen metabolism, and thus possibly provide insight into novel and viable cardioprotective interventions.

7.5 p38 MAPK, LKB1, and AMPK

LKB1 is a recently identified AMPK kinase (AMPKK) that is expressed in the rat heart, and activates AMPK by phosphorylating threonine-172 in catalytic α-subunits. Interestingly, LKB1 appears to be insensitive to hypoxia and ischemia, as these metabolic stresses are not sufficient to alter LKB1 activity [5,6]. This may suggest the existence of alternate, as yet unidentified AMPKK(s). Furthermore, the mechanism by which LKB1 activates AMPK may be related to the degree of interaction/association between the two kinases [7], rather, than an increase in LKB1 activity. The results presented in Chapters 4 and 5 suggest that p38 MAPK lies upstream of AMPK in mediating the effects of adenosine on glucose utilization in stressed hearts, however, do not assess the role or involvement of LKB1 in activating AMPK.

p38 MAPK is a proline directed serine/threonine protein kinase. Although, the catalytic threonine-172 residue of both AMPK α_1 and AMPK α_2 subunits does not reside in a p38 MAPK phosphorylation consensus sequence, and thus may rule out p38 MAPK as a direct AMPKK, whether or not p38 MAPK influences the interaction between LKB and AMPK is not known. Interestingly both AMPK α_1 and AMPK α_2 subunits contain several p38 MAPK phosphorylation consensus sequences (Fig. 7-1), as do the regulatory β_1, β_2, and γ_2 subunits (Fig7-2). Whether p38 MAPK is capable of phosphorylating AMPK catalytic and regulatory subunits within these consensus sequences, and promote the interaction/association between LKB1 and AMPK is not known. This facet of p38 MAPK-AMPK signaling can be assessed via *in vitro* kinase assays, as well as with immunoprecipation experiments, using p38 MAPK activators and inhibitors.

7.6 Translating Experimental Cardioprotective Strategies into Clinical Practice

Despite more than 30 yrs for IR of research into the pathophysiology of myocardial IR injury, and numerous promising experimental pharmacological therapies, very few pharmacological interventions have made the transition from the experimental setting to the clinical arena [8,9]. This inability to successfully translate experimental findings into clinically useful therapies may be attributed to multiple factors, the most apparent being species differences. Perhaps more important is the use of animal models that do not effectively recapitulate the clinical setting. For example, nutritional and hormonal states, as well as the presence of other co-morbidities also likely contribute to the lack of successful translation from experimental models to the clinical setting. In addition, the timing of drug administration in the experimental setting is very well controlled, with pharmacological intervention usually occurring prior to the onset of ischemia, as potential cardioprotective effects may be attenuated or lost when the drug is administered only during reperfusion. More careful separation of "anti-ischemic" vs "anti-reperfusion injury" is required. Treatment prior to the onset of ischemia in the clinical setting is virtually impossible (with the exception of planned cardiac surgery), and any useful cardioprotective therapy for AMI must be administered at the time of, or immediately prior to, reperfusion initiated by thrombolysis or percutaneous transluminal coronary angioplasty. As almost all experimental models of myocardial IR injury have an inherent window of viability, whether *in vitro* or *in vivo*, it remains unclear whether the numerous potential cardioprotective strategies already identified actually attenuate IR injury or merely delay the development of injury without altering overall magnitude.

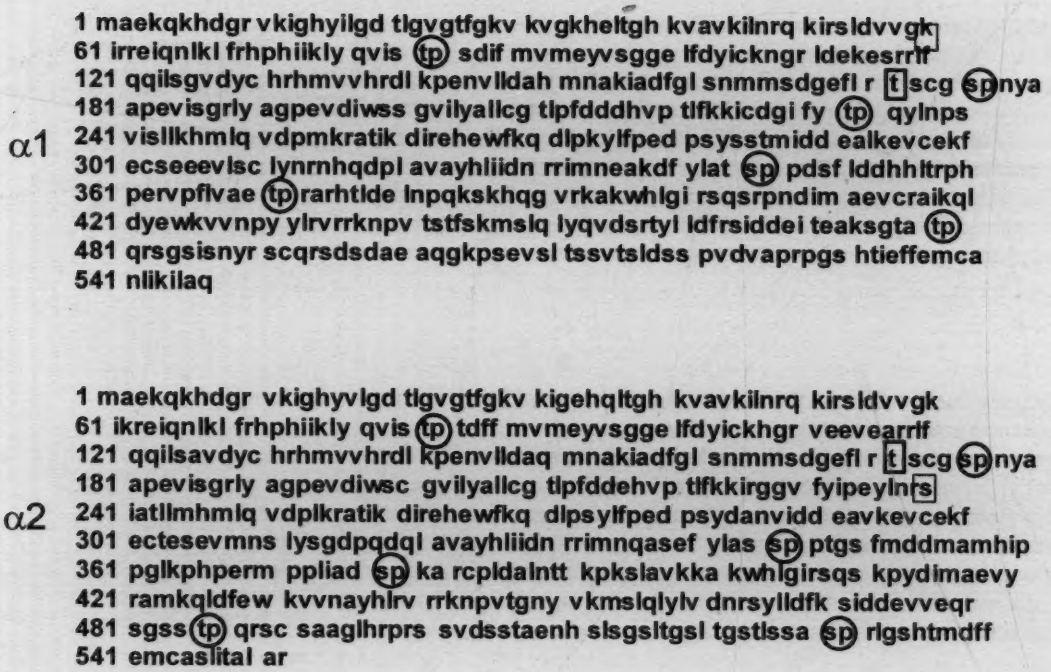
Thus, experimental models, and protocols must be developed to more effectively recapitulate clinical scenarios of myocardial IR injury in order to foster the translation of experimental findings into clinical practice.

Hypothermic cardioplegia is one cardioprotective modality that is useful in the clinical setting for elective cardiac surgery and transplantation. The experimental model of hypothermic cardioplegia and IR injury that was utilized in Chapter 2 effectively recapitulates the clinical scenario, where preservation times for cardiac allografts remain less than eight hours. This model also allows the examination of pharmacological interventions to enhance the protective properties of hypothermic cardioplegia. Moreover, it allows a realistic examination of the timing of pharmacological intervention during the differing phases of organ procurement, storage, and transplantation. As reported above, additional cardioprotection may be achieved with pharmacological intervention during storage (inhibition of ERK 1/2), and during working mode reperfusion (inhibition or p38 MAPK). The experimental model utilized in Chapters 3 and 4 allowed the characterization of mechanisms that potentially contribute to the loss of adenosine-induced cardioprotection, particularly the uncoupling of glucose metabolism. This was an important objective as adenosine is already an approved drug for the treatment of paroxysmal supraventricular tachycardia (PSVT), but clinical evaluation has shown that its cardioprotective effectiveness in diseased human hearts to be much less than predicted from animal studies. The studies described in this thesis have examined potential mechanisms underlying this alteration in cardioprotective effectiveness and have implicated alterations in signaling by the stress kinases, p38 MAPK and AMPK. Importantly, the results presented in Chapter 5 indicate that the loss of adenosine-induced

cardioprotection can be rescued by p38 MAPK or AMPK inhibition, and that this is accompanied by salutary alterations in glucose metabolism. These data indicate that p38 MAPK and AMPK regulate myocardial energy substrate metabolism, and suggests that these kinases are novel pharmacological targets to limit or ameliorate IR injury.

Figure 7-1. Primary amino acid sequences of the α_1 and α_2 catalytic subunits of AMPK. The critical threonine-172 residue is highlighted in the rectangles. p38 MAPK phosphorylation consensus sequences are highlighted in circles.

Figure7-1


1 maekqkhdgr vkighyilgd tlgvgtfgkv kvgkheltgh kvavkilnrq kirsldvvgk
61 irreiqnlkl frhphiikly qvis (tp) sdif mvmeyvsgge lfdyickngr ldekesrrlf
121 qqilsgvdyc hrhmvvhrdl kpenvlldah mnakiadfgl snmmsdgefl r [t] scg (sp) nya
181 apevisgrly agpevdiwss gvilyallcg tlpfdddhvp tlfkkicdgi fy (tp) qylnps
241 vislikhmlq vdpmkratik direhewfkq dlpkylfped psysstmidd ealkevcekf
301 ecseeevlsc lynrnhqdpl avayhliidn rrimneakdf ylat (sp) pdsf lddhhltrph
361 pervpflvae (tp) rarhtlde lnpqkskhqg vrkakwhlgi rsqsrpndim aevcraikql
421 dyewkvvnpy ylrvrrknpv tstfskmslq lyqvdsrtyl ldfrsiddei teaksgta (tp)
481 qrsgsisnyr scqrsdsdae aqgkpsevsl tssvtsldss pvdvaprpgs htieffemca
541 nlikilaq
α1

1 maekqkhdgr vkighyvlgd tlgvgtfgkv kigehqltgh kvavkilnrq kirsldvvgk
61 ikreiqnlkl frhphiikly qvis (tp) tdff mvmeyvsgge lfdyickhgr veevearrlf
121 qqilsavdyc hrhmvvhrdl kpenvlldaq mnakiadfgl snmmsdgefl r [t] scg (sp) nya
181 apevisgrly agpevdiwsc gvilyallcg tlpfddehvp tlfkkirggv fyipeylnrs
241 iatllmhmlq vdplkratik direhewfkq dlpsylfped psydanvidd eavkevcekf
301 ectesevmns lysgdpqdql avayhliidn rrimnqasef ylas (sp) ptgs fmddmamhip
361 pglkphperm ppliad (sp) ka rcpldalntt kpkslavkka kwhlgirsqs kpydimaevy
421 ramkqldfew kvvnayhlrv rrknpvtgny vkmslqlylv dnrsylldfk siddevveqr
481 sgss (tp) qrsc saaglhrprs svdsstaenh slsgsltgsl tgstlssa (sp) rlgshtmdff
541 emcaslital ar
α2

Figure 7-2. Primary amino acid sequences of the AMPK regulatory β_1, β_2, and γ_2 subunits. p38 MAPK phosphorylation consensus sequences are highlighted in circles.

Figure 7-2

β1

```
  1 mgntsseraa lerqaghk(tp)rrdssggtkd gdrpkilmds pedadifhte emkapekeef
 61 lawqhdlevn ekapaqarpt vfrwtgggke vylsgsfnnw sklpltrsqn nfvaildlpe
121 gehqykffvd gqwthd(ps)ep ivtsqlgtvn niiqvkktdf evfdalmvds qkcsdvsels
181 s(sp)pgpyhqe pyiskpeerf kappilpphl lqvilnkdtg iscdpallpe pnhvmlnhly
241 alsikdgvmv lsathrykkk yvttllykpi
```

β2

```
  1 mgnttservs gerhgakaar aeggghgpgk ehkimvgstd d(ps)vfslpds klpgdkefvp
 61 wqqdlddsvk ptqqarptvi rwseggkevf isgsfnnwst kiplikshnd fvaildlpeg
121 ehqykffvdg qwvhd(ps)epv vtsqlgtinn lihvkksdfe vfdalkldsm essetscrdl
181 ss(sp)pgpygq emyvfrseer fksppilpph llqvilnkdt niscdpallp epnhvmlnhl
241 yalstkdsvm vlsathrykk kyvttllykpi
```

γ2

```
  1 mleklefqee edsesgvymr fmrshkcydi vptsskivvf dttlqvkkaffalvangvra
 61 aplweskkqs fvgmltitdf inilhryyks pmvqiyelee hkietwrely lqetfkplvn
121 l(sp)daslfda vysliknkih rlpvidpisg nalyilthkr ilkflqlfms dmpkpafmkq
181 nldelgigty hniafihpnt piikalnifv errisalpvv desgkvvdiy skfdvinlaa
241 ektynnldit vtqalqhrsq yfegvvkcsk letletivdr ivraevhrlv vvneadsivg
301 iislsdilqa liltpagakq ketete
```

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